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Abstract of the Dissertation

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Nucleotide excision repair (NER) is a versatile DNA repair pathway that enables cells to eliminate a plethora of helix-distorting lesions caused by different environmental agents. Versatility and specificity in NER are achieved through the sequential and highly coordinated actions of at least 30 polypeptides that detect the lesion and excise a damage-containing oligonucleotide followed by repair synthesis and ligation events to restore the DNA sequence to its original state. Dual incision 5' to the lesion by XPF-ERCC1 and 3' to the lesion by XPG results in the removal of a lesion-containing oligonucleotide of about 30 nucleotides in length. This step leads to the formation of a single-stranded DNA (ssDNA) gap, which is filled in by the repair synthesis machinery. We addressed the question of how dual incision and repair synthesis are coordinated to avoid the exposure of potentially harmful ssDNA intermediates. Using catalytically inactive mutants of XPF-ERCC1 and XPG, we show that the 5' incision by XPF-ERCC1 precedes the 3' incision by XPG and that the initiation of repair synthesis does not require the catalytic activity of XPG. We propose that a defined order of dual incision and repair synthesis steps is important for the smooth progression through the NER pathway.

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Chapter 1

General Introduction

DNA DAMAGE AND REPAIR

The repair of damaged DNA is of crucial importance to protect the genome from numerous endogenous and environmental agents that cause alterations in DNA. DNA damage can have several consequences for a cell: first, it can activate checkpoint signaling and lead to cell cycle arrest. Second, it can cause apoptosis (cell death) through inhibition of transcription, replication and chromosome segregation. Finally, if the damage is not repaired and the cell with the damage is not removed from the population, it can cause mutations and chromosomal aberrations, leading to cancer, ageing or inborn disease (Hoeijmakers, 2001).

There are several types of DNA damage caused by a variety of agents. To deal with this multitude of lesions, several pathways specialized for the repair of a particular type of DNA damage have evolved. Lesions caused by normal cellular metabolism, such as those resulting from reactive oxygen species, methylation, deamination (for example uracil, abasic sites and 8-oxoguanine) as well as single-strand breaks caused by ionizing radiation, are repaired by base

excision repair (BER) (Schärer, 2003). This pathway is initiated by a DNA glycosylase that hydrolyzes the N-glycosidic bond between the base and the deoxyribose sugar. The AP endonuclease APE1 is the second enzyme in the pathway and hydrolyzes the phosphodiester bond 5' to the abasic site to generate a nick. In the main BER pathway, called short-patch repair, DNA polymerase β then incorporates a single nucleotide and removes the abasic site by virtue of its AP lyase activity. If BER is initiated by a bifunctional DNA glycosylase/AP lyase, this AP lyase activity of polymerase β may not be required. The nick in DNA is finally sealed by DNA ligase III/XRCC1 (X-ray repair cross-complementing group 1). A second, minor pathway for the repair of abasic sites in BER, termed long-patch repair, relies on DNA polymerase δ/ε and associated replication factors to introduce 2-6 nucleotides past the abasic site, the flap endonuclease FEN-1 to excise the short oligonucleotide overhang generated, and DNA ligase I to seal the nick (Schärer, 2003).

Nucleotide excision repair (NER) deals with the wide range of helix-distorting lesions caused by UV light or various chemical mutagens. Examples of the lesions processed by NER are cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts (6-4PPs) and 1,3-intrastrand d(GpTpG) cisplatin-DNA crosslinks. About 30 proteins participate in NER (Aboussekhra et al., 1995; Araujo et al., 2000; Mu et al., 1995): XPC-HR23B, the ten subunits of the basal transcription factor TFIIH, the single-stranded DNA binding protein RPA and XPA are involved in damage recognition and the opening of the DNA around the lesion. XPF-ERCC1 and XPG are two structure-specific endonucleases that carry out

incisions 5' and 3' to the lesion, respectively. The DNA polymerases δ and/or ε, assisted by the sliding clamp PCNA and the clamp loader RFC, fill in the repair gap to restore the original DNA sequence and DNA ligase I seals the nick. These proteins are required for the repair of the lesions throughout the entire genome in a process called global genome NER (GG-NER). A sub-pathway of NER, transcription-coupled NER (TC-NER), preferentially removes damage from the transcribed strand of active genes and is initiated through stalling of an elongating RNA polymerase at DNA lesions . TC-NER is not dependent on XPC-HR23B (Venema et al., 1991), but it requires additional proteins such as CSA and CSB that are not required for GG-NER (Svejstrup, 2002).

Mismatch repair (MMR) has specialized for replication errors in particular base-base mismatches and small insertions and deletions. Mismatch recognition in eukaryotes is mediated by one of two heterodimers, composed of MSH2 and MSH6 (MutSα) or MSH2 and MSH3 (MutSβ). MutSα is more abundant and participates in the repair of base/base mismatches and small strand misalignments of 1–2 nucleotides, whereas MutSβ is responsible for initiating the repair of larger loops. In the subsequent step, the mismatch bound MutSα or MutSβ recruits a heterodimer composed of MLH1 and PMS2 (PMS1 in yeast), referred to as MutLα. The MutSα/MutLα complex is then thought to translocate along the DNA contour in an ATP-dependent manner until it encounters a strand break, where it can load the exonuclease (exonuclease I) required for the degradation of the error-containing strand. It has been suggested that MMR is directed to the newly synthesized DNA strand by preexisting termini, such as

gaps between Okazaki fragments in the lagging strand or the 3′ terminus of the primer on the leading strand. This presupposes that the MMR process is closely coupled to replication. This would appear to be the case, as both MSH6 and MSH3 interact with proliferating cell nuclear antigen (PCNA), which is the processivity factor of replicating DNA polymerases (Jiricny, 2006).

Double-strand DNA breaks (DSBs) induced by X-rays, chemicals or during replication of single-strand breaks as well as during repair of interstrand crosslinks can be repaired by two different types of mechanism: homologous recombination (HR) and non-homologous end joining (NHEJ). Proteins encoded by genes in the *RAD52* epistasis group of *S. cerevisiae* (or their homologs in other eukaryotic organisms), including RAD51, RAD52, RAD54, RAD55, RAD57 and RAD59, are involved in DSB repair via homologous recombination. In higher eukaryotes HR is especially important in early development and in the late S and G2 phase of the cell cycle, when sister- chromatids are available. NHEJ predominates in G1, early S-phase and in terminally differentiated cells. NHEJ requires at least the DNA-end binding Ku complex (initially recognizes the DNA break), a protein kinase DNA-PKcs, which signals the presence of a break and activates repair proteins at the break, DNA-end processing enzymes (for example, Artemis), and the XRCC4–Ligase IV-Cernunnos/XLF complex, which re-ligates the broken DNA ends (Wyman and Kanaar, 2006).

NUCLEOTIDE EXCISION REPAIR (NER)

History

The history of NER began in 1874 when Moriz Kaposi described a disease that he named xeroderma pigmentosum (XP) (von Hebra and Kaposi, 1874). XP patients exhibit severe sensitivity to sunlight and have more than 1000-fold increased incidence of skin cancer in sun-exposed areas. A significant number of XP patients display progressive neurological degeneration (Bootsma et al., 1997). Most XP patients are defective in both GG-NER and TC-NER.

In 1936 another disease was reported that leads to UV sensitivity, Cockayne syndrome (CS) (Cockayne, 1936). CS patients have short stature, severe neurological abnormalities, bird-like faces and cataracts, but no predisposition to skin cancer. CS cells were originally characterized by a deficiency in TC-NER (Svejstrup, 2002). However, since most XP patients are also deficient in TC-NER, this defect is not sufficient to explain the severe symptoms displayed by CS patients. It is therefore believed that CS cells have also a mild global transcription defect (van Gool et al., 1997).

Lastly, in 1980 Price reported a disease termed trichothiodystrophy (TTD) characterized by sulfur-deficient brittle hair and scaling of the skin in addition to symptoms displayed in CS patients (Price et al., 1980). The disease is now known to be associated with mutations in genes coding for XPB, XPD and TTD-A, subunits of NER/transcription factor TFIIH (Bergmann and Egly, 2001; Giglia-Mari et al., 2004).

The beginnings of the elucidation of the NER pathway date to 1964 when two important observations were made: it was shown that thymine dimers are released in the form of short oligonucleotides from UV-irradiated DNA in bacteria (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964) and that DNA is synthesized *de novo* following UV irradiation in bacterial and human cells (Pettijohn and Hanawalt, 1964; Rasmussen and Painter, 1964). The latter phenomenon, referred to as "unscheduled DNA synthesis" (UDS), reflects the repair synthesis step of NER and is impaired in cells from XP patients (Cleaver, 1968). Restoration of UDS can be achieved by the fusion of cells from XP patients with defects in various XP genes (de Weerd-Kastelein et al., 1972) and has led to the identification of different complementation groups, designated XP-A to XP-G, each of them carrying mutations in genes coding for different NER proteins.

NER Substrates

Lesions recognized and processed by NER are very diverse, but can be roughly divided in two groups, depending on the agents causing them:

UV light causes mainly cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone adducts (6-4 PPs). CPDs are the major UV-induced DNA photoproducts, usually formed between adjacent thymine residues (TT). CPDs induce only a slight bending of the DNA helix with no significant alteration of the base pairing (Kim et al., 1995). 6-4 PPs are induced preferentially at TC, CC and TT nucleotides and produce a more pronounced distortion of the DNA

backbone (bending and unwinding) than CPDs, resulting in loss of base pairing at the site of the lesion (Kim et al., 1995).

Many electrophilic compounds react with DNA, especially with the N7 of guanine base, which is easily accessible (it is localized in the major groove of the DNA) and has high electron density. *cis*-Diaminodichloroplatinum (cisplatin) preferentially induces intrastrand N7-N7 crosslinks between adjacent purine bases: 1,2-d(GpG), 1,2-d(ApG) and 1,3-d(GpNpG). The 1,2-intrastrand crosslink produces a pronounced kink in the DNA, but retains almost intact base pairing and is a poor NER substrate. The 1,3-intrastrand adduct induces less DNA bending, but exhibits significant helix unwinding and disruption of base pairing in the vicinity of the cisplatin adduct and is a much better NER substrate (Huang et al., 1994; Jamieson and Lippard, 1999; Szymkowski et al., 1992; Zamble et al., 1996). Polycyclic aromatic hydrocarbons (like benzo[a]pyrene, present in cigarette smoke and automobile exhausts) and aromatic amines (like aminofluorene, AF, which was originally developed as an insecticide, but was found to be carcinogenic) undergo metabolic activation through cellular detoxification mechanisms into potent carcinogens which form different kinds of adducts on dG (Geacintov et al., 1997; Heflich and Neft, 1994).

There are two possible explanations for the remarkable diversity of lesions processed by NER: a) There is more than one protein involved in damage recognition/verification, and b) Not (only) the chemical alteration of the lesion is recognized by NER machinery, but rather the structural changes in the vicinity of lesion. Probably both are true. In 1997, Naegeli and co-workers proposed a

"bipartite substrate discrimination" model, according to which both chemical modification and a disruption of base pairing are needed for a lesion to be processed by NER (Hess et al., 1997b). That model explains higher repair rates of 6-4 PPs versus CPDs and 1,3-d(GpNpG) versus 1,2-d(GpG) lesions, but would also predict efficient repair of all the adducts that induce disruption of base pairing, which is not always the case (Buterin et al., 2000; Hess et al., 1997a; Yan et al., 2003). Later it was proposed that the amount of thermodynamic destabilization induced by DNA damage (including several parameters like base pairing disruption, DNA unwinding, bending and flexibility) is the key determinant for the damage recognition (Geacintov et al., 2002; Isaacs and Spielmann, 2004).

Molecular Mechanism of GGR

Damage Recognition: XPC-HR23B and XPE

Damage recognition in GGR has been a matter of considerable debate over the years. XPA (Jones and Wood, 1993), RPA (Burns et al., 1996; He et al., 1995; Schweizer et al., 1999), XPC-HR23B (Batty et al., 2000; Sugasawa et al., 1998; Sugasawa et al., 2001) and XPE (Fujiwara et al., 1999; Keeney et al., 1993; Reardon et al., 1993) all bind preferentially to damaged DNA, but none of them had enough specificity to be unequivocally identified as the initial damage recognition factor from the *in vitro* measurements of its binding affinities and specificities for damaged DNA.

Local UV irradiation followed by immunofluorescence, a technique developed several years ago (Mone et al., 2001), allowed the identification of the

damage-sensing factor *in vivo*. Studies using this technique showed that XPC accumulated at the sites of UV lesions in XPA deficient (XP-A) cells, while there was no recruitment of XPA to the UV-damaged DNA in XP-C cells. That demonstrated that XPC is required for the recruitment of XPA to the sites of UV damage and therefore XPC has to arrive at the lesion before XPA (Volker et al., 2001). Later biochemical studies have shown that XPC is required for the opening of the DNA around the lesion and subsequent recruitment of all the other NER factors in a reconstituted system (Riedl et al., 2003; Tapias et al., 2004). However, it is still poorly understood how XPC recognizes the lesion. Most of the XPC in cells is present in a complex with HR23B (Masutani et al., 1994) and this interaction is believed to stabilize the XPC protein and protect it from proteasomal degradation (Ng et al., 2003). The third member of the complex, centrin 2 (Araki et al., 2001), has been shown to stimulate NER reaction (Nishi et al., 2005), but its precise role is not well understood. *In vitro* studies have shown that XPC-HR23B binds preferentially to various types of damaged DNA and the complex showed greater affinity for lesions that induce more helical distortion (Batty et al., 2000; Sugasawa et al., 1998). It binds small bubble structures and damaged DNA with similar efficiency, arguing that it does not recognize the lesion itself, but rather a distortion in the DNA structure (Sugasawa et al., 2001). In agreement with this hypothesis, the less distorting CPDs are bound less specifically by XPC-HR23B and are less efficiently processed by NER than the more distorting 6-4 PPs (Batty et al., 2000; Szymkowski et al., 1993). However, when a mismatch was introduced opposite the CPD, binding affinity by HR23B-

XPC and processing by NER were significantly increased (Sugasawa et al., 2001). It has been proposed that XPE (Wittschieben and Wood, 2003), UV-damaged DNA binding factor (UV-DDB), consisting of two subunits, p127/DDB1 and p48/DDB2, could play a role in lesion detection for some less distorting DNA alterations. It was shown that XPE binds specifically to CPDs and bends the DNA around the lesion (Fujiwara et al., 1999; Tang and Chu, 2002) and that is required for the recruitment XPC to CPD containing sites *in vivo* (Fitch et al., 2003b), stimulating repair of these lesions (Wakasugi et al., 2002). The details of how XPE interacts with lesion in NER remain poorly understood.

DDB1 and DDB2 were found to be part of an ubiquitin ligase complex containing Roc1 and Cul4A (Groisman et al., 2003). Associated with the DDB-Cul4A E3 ligase complex is COP9 signalosome (CSN), which suppresses ubiquitylation by the Cul4A ligase. After binding to UV-damaged DNA, CSN dissociates from the E3 ligase complex leading to polyubiquitylation of DDB2 and its subsequent degradation by the proteasome (Fitch et al., 2003a; Groisman et al., 2003; Rapic-Otrin et al., 2002).

Recently, two independent studies described the UV-dependent ubiquitylation of XPC by the same ubiquitin ligase complex (Sugasawa et al., 2005; Wang et al., 2005). Contrary to the fate of polyubiquitylated DDB2, polyubiquitylated XPC is not degraded (Sugasawa et al., 2005) and displays an increased affinity for both damaged and undamaged DNA. It still remains to be established how those and other posttranslational modifications influence the assembly of NER machinery.

Open Complex Formation: TFIIH

The next factor to arrive to the site of the lesion is TFIIH, a ten-subunit complex, composed of a core sub-complex (XPB, XPD, p62, p52, p44, p34, p8) and a cyclin-activating kinase sub-complex (CAK: Cdk7, cyclin H, Mat1). It was originally isolated as a basal transcription factor (Gerard et al., 1991), but with the discovery that XPB and XPD are parts of TFIIH, its involvement in NER was uncovered: XPB and XPD are helicases with 3'→5' and 5'→3' directed DNA unwinding activity, respectively, and are involved in forming open DNA structures in both transcription and NER (Schaeffer et al., 1994; Schaeffer et al., 1993). In NER, both XPB and XPD are required for the opening of the DNA around a lesion (Evans et al., 1997), while only XPB helicase activity is needed for transcription *in vitro* (Tirode et al., 1999). XPB is essential for transcription initiation and promoter escape (Coin et al., 1999), steps regulated by phosphorylation in the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNA Pol II) mediated at least partially by the Cdk7 kinase of TFIIH (Svejstrup et al., 1996). Although the helicase activity of XPD is dispensable for transcription, it facilitates optimal transcription by anchoring the CAK sub-complex to the core TFIIH (Tirode et al., 1999). Interestingly, it has been shown recently that the ATPase, but not the helicase activity of XPB is used for damaged DNA opening in NER, in combination with the helicase activity of XPD (Coin et al., 2007).

TFIIH is recruited to the site of the DNA damage through its interaction with XPC-HR23B (Drapkin et al., 1994; van der Spek et al., 1996; Volker et al., 2001; Yokoi et al., 2000) in an ATP-independent manner (Araujo et al., 2001; Riedl et al., 2003; Uchida et al., 2002). At this stage, assembly of TFIIH does not greatly modify the DNA opening initially stabilized by XPC-HR23B (Tapias et al., 2004). In a second ATP-dependent step, TFIIH extends the DNA opening to allow the entry of subsequent NER factors. This is the first catalytic and likely irreversible step of the NER reaction, so called “kinetic proofreading” (Reardon and Sancar, 2003, 2004). It is interesting to speculate that the damage verification and the determination of which strand contains the lesion could be fulfilled by the blockage of one of the two helicases at the site of the lesion (Naegeli et al., 1992; Naegeli et al., 1993; Sung et al., 1994). Such an ATP-dependent scanning recognition mechanism could explain both the substrate versatility of NER and would be consistent with the notion that both a chemical modification and a distortion of DNA helix are required for efficient processing by NER (Hess et al., 1997b). Studies with pre-melted DNA substrates containing a site-specific lesion revealed that DNA unwinding is not the only function of TFIIH in excision repair. Such pre-melted bubbles still required the presence of TFIIH for efficient further processing, indicating an additional, probably structural role for TFIIH in repair (Mu and Sancar, 1997). For example, TFIIH is required for recruitment of XPG to NER complexes (Dunand-Sauthier et al., 2005; Volker et al., 2001).

A newly identified, tenth subunit of TFIIH, p8 (Giglia-Mari et al., 2004), might also have an important role in determining which substrates will be processed further. p8 is present in two distinct kinetic pools: one bound to TFIIH, and a free fraction that shuttles between the cytoplasm and nucleus. After induction of NER-specific DNA lesions, the equilibrium between these two pools shifts towards a more stable association of p8 with TFIIH. Modulating transcriptional activity in cells does not induce a similar shift in this equilibrium. Surprisingly, DNA conformations that only provoke an abortive-type of NER reaction do not result in a more stable incorporation of p8 into TFIIH (Giglia-Mari et al., 2006). It has also been shown that p8 triggers DNA opening by stimulating XPB ATPase activity together with XPC-HR23B. Such opening is needed for the recruitment of XPA to the site of the damage (Coin et al., 2006). Together, these data suggest an important role for TFIIH in DNA damage verification and assembly of mature NER complexes.

Assembly of the Preincision Complex

After initial opening of the DNA helix, XPA, RPA and XPG join the complex and XPC-HR23B dissociates (Riedl et al., 2003; Wakasugi and Sancar, 1998). The order of association of XPA, RPA and XPG with TFIIH is not clear, but it is likely that they are recruited to the NER complex independently of each other (Rademakers et al., 2003).

XPA was originally thought to be the initial damage recognition factor (Asahina et al., 1994; Jones and Wood, 1993; Kuraoka et al., 1996), possibly together with RPA (Buschta-Hedayat et al., 1999; He et al., 1995; Li et al.,

1995a; Wakasugi and Sancar, 1999). When XPC-HR23B was assigned that role, XPA was believed to perform damage verification (Lee et al., 2003). The most recent studies demonstrated that XPA has much higher affinity for binding unusual kinked DNA structures than DNA lesions and as such might be confirming the existence of DNA lesions indirectly, by probing for distorted DNA as a result of a lesion (Camenisch et al., 2006; Missura et al., 2001). Apart from interacting with DNA, XPA interacts also with XPC-HR23B (You et al., 2003), RPA (Ikegami et al., 1998; Matsuda et al., 1995; Saijo et al., 1996), TFIIH (Li et al., 1998; Park et al., 1995b) and ERCC1 (Li et al., 1995b; Nagai et al., 1995; Park and Sancar, 1994) which points to a possible role in proper assembly of NER complexes.

RPA, a ssDNA-binding protein (for a review see (Bochkarev and Bochkareva, 2004)), has essential roles in DNA replication and recombination as well as in NER. It was found to be required both for the dual incision (Coverley et al., 1992; Guzder et al., 1995; Mu et al., 1995) and repair synthesis steps of NER (Coverley et al., 1991; Shivji et al., 1995). The largest subunit of RPA, RPA70, was shown to bind ssDNA with a defined orientation with respect to 5' and 3' polarity (Bochkarev et al., 1997) and binding of RPA to the undamaged ssDNA strand is likely to be involved in establishing the polarity of the NER complex (de Laat et al., 1998b).

Recruitment of XPG to NER complexes is dependent on TFIIH (Volker et al., 2001) and XPG is probably recruited via a direct protein-protein interaction with TFIIH (Araujo et al., 2001; Iyer et al., 1996). At this stage it contributes to

enlarging of the DNA opening initiated by XPC-HR23B and TFIIH and stabilization of the pre-incision complex (Evans et al., 1997; Mu et al., 1997; Tapias et al., 2004). In agreement with a role of XPG in the assembly of the NER pre-incision complex, XPG also interacts with RPA (He et al., 1995).

Recruitment of XPF-ERCC1 and Dual Incision

XPF-ERCC1 is the last repair factor that joins the pre-incision complex (Riedl et al., 2003; Wakasugi and Sancar, 1998) and its recruitment is dependent on XPA (Volker et al., 2001). Direct protein-protein interaction between XPA and ERCC1 (Li et al., 1994; Nagai et al., 1995; Park and Sancar, 1994; Saijo et al., 1996) is likely the way of XPF-ERCC1 recruitment as abolishment of this interaction results in reduced NER activity *in vitro* and *in vivo* (Li et al., 1995a).

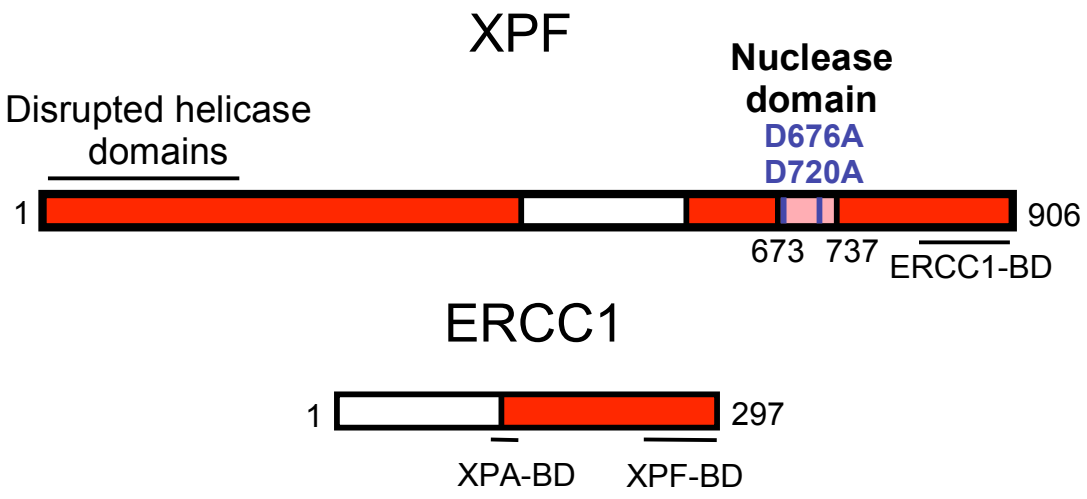

Fig.1. Schematic Representation of the XPF-ERCC1 Endonuclease. Domains participating in protein-protein interactions in XPF (ERCC1-BD) and ERCC1 (XPA-BD and XPF-BD) as well as the nuclease domain of XPF (with the important catalytic residues, mutations of which abolish nuclease activity) are indicated. Abbreviations: BD, binding domain.

XPF-ERCC1 (Figure 1) is a structure-specific endonuclease that is essential for dual incision in NER as it cleaves the DNA 5' to the lesion (Sijbers et

al., 1996). XPF and ERCC1 form a stable heterodimer *in vitro* (Biggerstaff et al., 1993; Park et al., 1995a) and *in vivo* (Sijbers et al., 1996; van Vuuren et al., 1993), which involves interaction of the C-termini of both proteins (de Laat et al., 1998c). XPF-ERCC1 incises a variety of DNA substrates including bubbles, stem-loops, splayed-arms and flap substrates (Bessho et al., 1997; de Laat et al., 1998a; Enzlin and Schärer, 2002; Sijbers et al., 1996). Incisions in these artificial substrates are always made in one strand of DNA duplex on the 5'-side of the junction with ssDNA, consistent with its cleavage polarity in NER (Sijbers et al., 1996). It was shown that binding of RPA to ssDNA could stimulate XPF-ERCC1 endonuclease activity on model substrates when the factors were positioned with a polarity reflective of the one found in NER reaction (Bessho et al., 1997; de Laat et al., 1998b; Matsunaga et al., 1996).

The active site of XPF-ERCC1 resides in the C-terminal half of XPF and consists of a conserved IERKX_3D motif, which is also present in other nucleases of the same family (Aravind et al., 1999; Enzlin and Schärer, 2002). Several point mutations in that region (for example, D676A and D720A) completely abolish its nuclease and NER activity without an effect on its DNA binding (Enzlin and Schärer, 2002; Tapias et al., 2004; Enzlin and Schärer, unpublished results).

XPG is the second structure-specific endonuclease in NER and it cuts 3' to the lesion (O'Donovan et al., 1994a). It is also active on various artificial DNA structures (splayed arm, bubble, stem loop and flap substrates) (Cloud et al., 1995; Habraken et al., 1994; O'Donovan et al., 1994a; O'Donovan et al., 1994b), all of which have a junction of single-stranded and duplex DNA and XPG cleaves

at the DNA junction on the 3' side of the ssDNA, consistent with the cleavage polarity in NER (O'Donovan et al., 1994a).

XPG (Figure 2) belongs to the FEN-1 family of structure-specific nucleases (Lieber, 1997; Suck, 1997). Sequence similarities among these proteins are largely confined to two regions, a N-terminal (N-region) and an internal region (I-region) (Harrington and Lieber, 1994), which contain highly conserved acidic residues essential for nuclease activity. Point mutations changing some of those residues to alanine (like D77A, E791A and D812A) abolish XPG nuclease and NER activity (Constantinou et al., 1999; Wakasugi et al., 1997). The unique feature of XPG is the "spacer region", over 600 amino acids separating its N and I domains, not present within other members of FEN-1 family (Scherly et al., 1993). The spacer region has been shown to contribute to the interaction with TFIIH and substrate specificity of XPG, enabling the cleavage of bubble substrates that are not processed by FEN-1 (Dunand-Sauthier et al., 2005; Hohl et al., 2007; Iyer et al., 1996; Thorel et al., 2004).

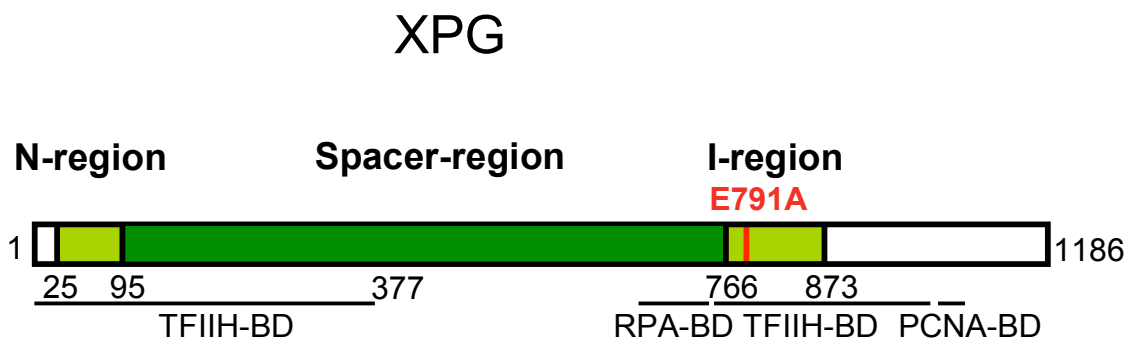

Fig.2. Schematic Representation of the XPG Endonuclease. Domains participating in protein-protein interactions (TFIIH-BD, RPA-BD, PCNA-BD), nuclease domain consisting of the N-region and I-region (with the important catalytic residue, E791, mutation of which to alanine abolishes nuclease activity) and the spacer-region (important for protein-protein interactions and substrate specificity) are indicated. Abbreviations: BD, binding domain.

When both endonucleases are recruited to the site of the damage, the dual incision takes place, releasing a damage-containing oligonucleotide of 24-32 nucleotides (de Laat et al., 1999).

Repair Synthesis

The gap, which is the result of the dual incision, has to be filled in by repair synthesis. The requirement for PCNA (Aboussekhra and Wood, 1995; Nichols and Sancar, 1992; Shivji et al., 1992), DNA polymerases δ (Hunting et al., 1991; Nishida et al., 1988; Zeng et al., 1994) and ε (Shivji et al., 1995) and RPA (Coverley et al., 1991) in repair synthesis during NER was recognized early on and RPA, PCNA, RFC, DNA polymerase δ or ε and DNA ligase I were shown to be necessary and sufficient for repair synthesis *in vitro* (Araujo et al., 2000; Shivji et al., 1995). All of those proteins have been characterized extensively in the context of DNA replication: RFC is a pentameric clamp loader, which catalyzes the ATP-dependent loading of PCNA to DNA near 3' termini of primers (Majka and Burgers, 2004). PCNA is a homotrimeric protein that forms a ring-shaped clamp (Krishna et al., 1994), which can slide along the DNA. It interacts with DNA polymerase to ensure processive replication (Miyata et al., 2004). DNA polymerases δ and ε are replicative polymerases and belong to the B family of DNA polymerases. They are able to replicate DNA at high speed, with high processivity and with a very low error rate. High fidelity is achieved by the active sites of these polymerases having stringent requirements, matching the incoming nucleotide to the template base by the appropriate Watson-Crick base pairing. In

addition, the 3'-5' exonucleases associated with replicative polymerases remove any base that might be mis-inserted (Lehmann, 2006).

RPA is the only protein needed for both steps of NER reaction, dual incision and repair synthesis, and as such it might be responsible for coupling of the two steps as it is involved in the recruitment of PCNA (Gomes and Burgers, 2001; Riedl et al., 2003) and RFC (Yuzhakov et al., 1999). Another factor that stays bound to the incised DNA longer than other NER proteins is XPG (Riedl et al., 2003). Through its interaction with PCNA (Gary et al., 1997; Miura et al., 1996) it might contribute to the coordination of the two steps (Gary et al., 1997). Interestingly, PCNA has been shown to stimulate the double incision in NER (Nichols and Sancar, 1992), again arguing for the tight coupling of the two steps as was most clearly demonstrated by similar kinetics of the excision and repair synthesis steps (Riedl et al., 2003).

The current model for NER reaction based on the data presented in this chapter is summarized in Figure 3.

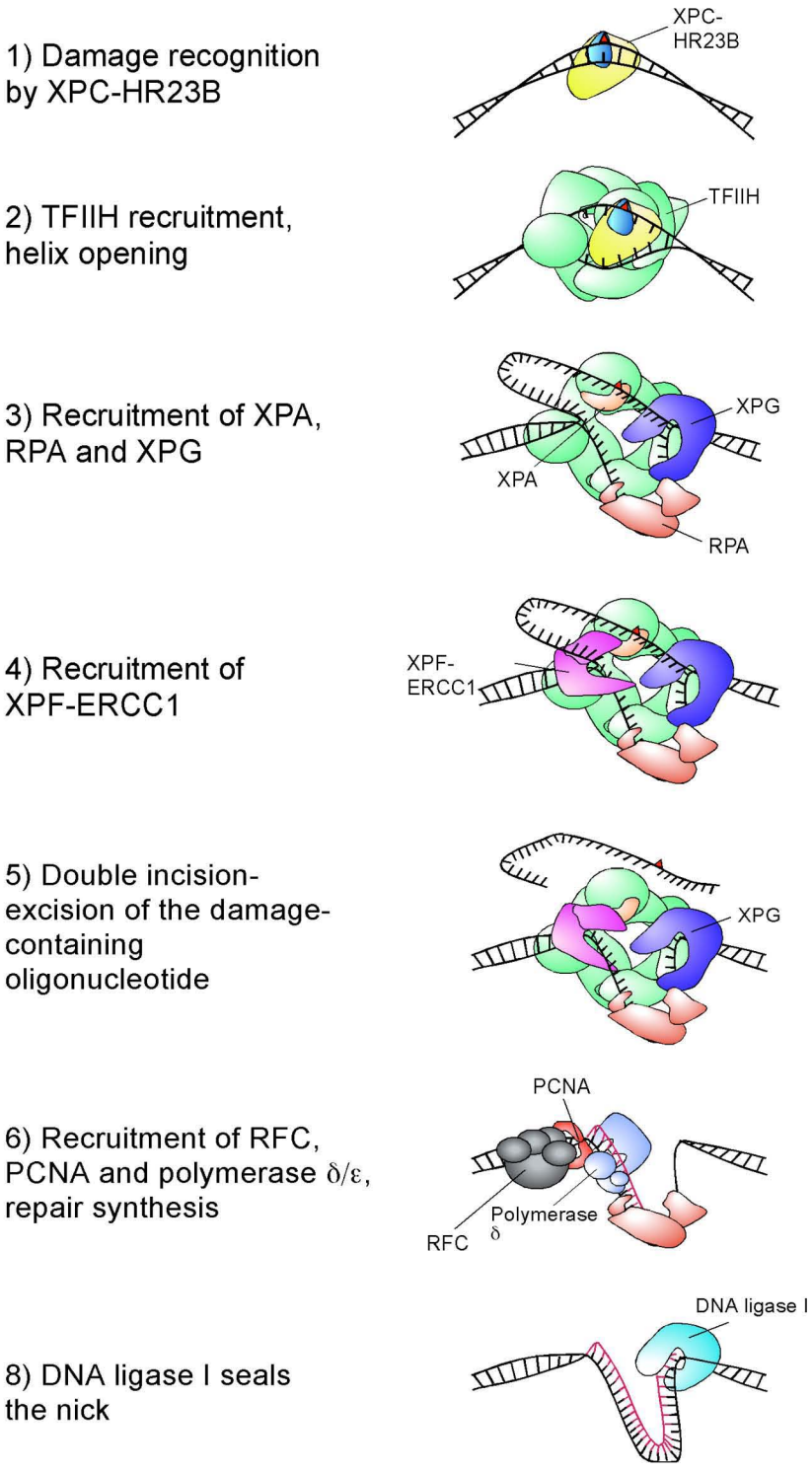

Fig.3. Current Model of the Nucleotide Excision Repair Pathway. Proteins participating in each step are indicated.

A model has been proposed in which 5' incision by XPF-ERCC1 occurs first, generating a free 3' hydroxyl end on the DNA, which may be used to initiate repair synthesis, followed by a conformational change in the multiprotein/DNA complex that will now allow XPG to exert its catalytic activity (Gillet and Schärer, 2006). This would ensure a smooth transition to the repair synthesis without formation of potentially recombinogenic and mutagenic ssDNA gaps at the place where the lesion-containing oligonucleotide was excised. Two observations are in line with this hypothesis: first, one study analyzed incision patterns in reconstituted NER reactions using wild type and catalytically inactive XPF-ERCC1 and found that efficient 3' incision occurred only in the presence of catalytically active XPF-ERCC1 (Tapias et al., 2004). Second, because XPG has distinct requirements for binding and cleaving DNA (Hohl et al., 2003), the incision by XPF-ERCC1 might bring the conformational change needed to trigger efficient excision by XPG. The main objective of the work described in this dissertation was to test this model.

Chromatin Reassembly

After repair synthesis and ligation, chromatin that was disrupted for the NER reaction to take place needs to be restored. Histone chaperones (Loyola and Almouzni, 2004) are likely to be involved in that process and the chromatin assembly factor 1 (CAF-1) represents an attractive candidate for this task. CAF-1 is a conserved nuclear complex consisting of p150, p60 (Kaufman et al., 1995) and p48 subunits (Verreault et al., 1996) in human cells. The recent discovery of its specific association with the replicative histone variant H3.1 in human cells

(Tagami et al., 2004) has offered novel insights into a potential role of CAF-1 for the establishment and/or maintenance of histone variant identity in specific chromatin domains. CAF-1 has the unique ability to promote chromatin assembly *in vitro* in a DNA synthesis-dependent manner on replicating (Smith and Stillman, 1989) and newly repaired DNA (Gaillard et al., 1996). Furthermore, it is recruited to UV-damaged chromatin *in vivo* in an NER-dependent manner (Green and Almouzni, 2003) indicating an involvement in a late stage of NER. Interestingly, CAF-1 recruitment has been shown to depend on PCNA (Green and Almouzni, 2003; Martini et al., 1998; Moggs et al., 2000), strengthening the hypothesis of a tight coordination between the repair synthesis and chromatin reassembly steps. It was shown recently that new H3.1 histones are incorporated at repair sites *in vivo* and that this histone deposition is dependent on nucleotide excision repair. CAF-1 was shown to be directly involved in the histone deposition process (Polo et al., 2006).

TRANSLESION SYNTHESIS

If damage is not removed from the DNA in time before a cell enters S-phase, it will block a replicating DNA polymerase. Cells have developed another defense mechanism to prevent the deleterious effects of stalled or incomplete replication. They use specialized DNA polymerases to carry out translesion synthesis past damage sites. Most of those polymerases are members of the Y-family (Ohmori et al., 2001). They are slower than replicative polymerases and operate with low processivity and fidelity. Their active site adopts much more

open structure than in replicative polymerases, which makes them less stringent so that they can accommodate altered bases in their active sites. A crystal structure of DNA polymerase eta in complex with CPD-containing DNA has shown how this may be accomplished (Ling et al., 2003).

DNA polymerase eta (polη) was discovered in mammalian cells as the protein deficient in the variant form of xeroderma pigmentosum (XP-V) (Johnson et al., 1999; Masutani et al., 1999). XP-V patients have normal levels of NER, but exhibit problems in replicating their DNA after UV irradiation (Lehmann et al., 1975). Polη is able to replicate past a CPD as efficiently as past undamaged bases *in vitro*, and in the majority of cases it inserts correct bases (Masutani et al., 2000; McCulloch et al., 2004). Polη, however, cannot bypass a 6-4 PP and there is some evidence that Rev1 and B-family DNA polymerase ζ are required for translesion synthesis past this lesion (Gibbs et al., 2005; Nakajima et al., 2004). All the Y-family polymerases are localized in the nucleus and during S-phase, polη, ι and Rev1 relocate to replication factories (Kannouche et al., 2003), where they colocalize with PCNA and other proteins involved in DNA replication. When the replication machinery is blocked, PCNA becomes mono-ubiquitinated activating the so called “polymerase switch” from a replicative to a translesion DNA polymerase (Hoege et al., 2002). The mechanism of how this switch operates was elucidated in 2005, after the discovery of novel ubiquitin-binding domains in DNA polymerases η, ι, Rev1 and κ (Bienko et al., 2005). Polymerases η, ι, and κ all have classical PCNA-binding “PIP” motifs, and have been shown to bind PCNA *in vitro* (Haracska et al., 2001; Vidal et al., 2004), but

not *in vivo*, suggesting that interactions are weak. Mono-ubiquitination of PCNA increases its affinity for polη and that interaction could be detected in cell extracts (Kannouche et al., 2004; Watanabe et al., 2004). The interaction is dependent on the novel ubiquitin-binding domains, which were shown to bind to ubiquitin in the case of polη and polι (Bienko et al., 2005). Thus, the combination of binding to ubiquitinated PCNA via both the PIP motif and the ubiquitin-binding domain strengthens the interactions between the polymerases and PCNA, facilitating their recruitment to the stalled fork and the polymerase switch.

PREVIEW

The topic of this dissertation is the analysis of late steps of the nucleotide excision repair pathway. The main focus of the work presented is the investigation of the events following the recruitment of the pre-incision complex, leading to the elucidation of the mechanism coordinating the two distinct steps in the NER reaction: dual incision and repair synthesis (Chapter 2). Additionally, the effect of early (XPA and RPA) and late (PCNA) NER factors on the activity of the two endonucleases, XPF-ERCC1 and XPG, has been analyzed (Chapter 3).

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Chapter 2

Coordination of Dual Incision and Repair Synthesis in Human Nucleotide Excision Repair

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ACKNOWLEDGMENTS

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SUMMARY

Nucleotide excision repair (NER) requires the coordinated sequential assembly of the involved proteins at sites of DNA damage. Following damage recognition, dual incision 5' to the lesion by XPF-ERCC1 and 3' to the lesion by XPG results in the removal of a lesion-containing oligonucleotide of about 30 nucleotides followed by repair synthesis to fill in the resulting single-stranded DNA gap. We addressed the question of how dual incision and repair synthesis are coordinated to avoid the exposure of potentially harmful single-stranded DNA intermediates. Using catalytically inactive mutants of XPF-ERCC1 and XPG, we show that the 5' incision by XPF-ERCC1 precedes the 3' incision by XPG and that the initiation of repair synthesis does not require the catalytic activity of XPG. We propose that a defined order of dual incision and repair synthesis is important for the smooth progression through the NER pathway.

INTRODUCTION

Nucleotide excision repair (NER) is a versatile DNA repair pathway that enables cells to eliminate a plethora of helix-distorting lesions caused by different environmental agents. Versatility and specificity in NER are achieved through the sequential and highly coordinated actions of at least 30 polypeptides that detect the lesion and excise a damage-containing oligonucleotide followed by repair synthesis and ligation events to restore the DNA sequence to its original state (de Laat et al., 1999; Friedberg et al., 2005; Gillet and Schärer, 2006). A subpathway of NER, transcription-coupled NER (TC-NER), preferentially removes damage from the transcribed strand of active genes and is initiated through stalling of an elongating RNA polymerase at DNA lesions (Hanawalt, 2001; Svejstrup, 2002). In bulk DNA, XPC-HR23B appears to be the initial sensor of DNA damage and is essential for the assembly of all subsequent NER factors in the process known as global genome NER (GG-NER) (Sugasawa et al., 1998; Sugasawa et al., 2001; Volker et al., 2001). TFIIH, the next factor to be recruited, is responsible for strand separation around the lesion (Evans et al., 1997b; Tirode et al., 1999; Wakasugi and Sancar, 1998), enabling XPA, RPA and XPG to join the complex. XPF-ERCC1, the subsequent factor to be recruited (Mu et al., 1997; Tapias et al., 2004), performs the incision 5' to the damage (Bardwell et al., 1994; Sijbers et al., 1996) while XPG cleaves 3' to the lesion (O'Donovan et al., 1994). An oligonucleotide of 24-32 nucleotides in length containing the lesion is then released and the resulting gap is filled by DNA

polymerase δ/ε (and/or possibly κ (Ogi and Lehmann, 2006)), replication factor C (RFC), PCNA, RPA and the nick is sealed by DNA ligase I (Shivji et al., 1995) to restore the original DNA sequence. At a higher level of organization, chromatin assembly factor 1 (CAF-1) has been implicated in the restoration of chromatin following the repair reaction (Green and Almouzni, 2003).

While many recent studies have been concerned with the mechanisms of damage recognition, less is known about the transition from dual incision to repair synthesis. Simple release of the oligonucleotide containing the damaged residue would result in the formation of a ssDNA gap, another deleterious DNA lesion with a key role in activating DNA damage signaling pathways (Shechter et al., 2004). It therefore appears likely that a mechanism ensuring the smooth transition between the dual incision and repair synthesis steps would have evolved. The similar kinetics of the damage removal and repair synthesis indeed suggests a coordination of these two events (Riedl et al., 2003). For repair synthesis to occur, the 5' incision by XPF-ERCC1 is required to generate a free 3' OH group for the polymerase reaction. By contrast, the 3' incision by XPG may not necessarily be needed to initiate polymerization. A temporal order of the two incisions with the 5' incision occurring first might therefore contribute to an efficient transition from dual incision to repair synthesis.

Analysis of the literature reveals that there is no consensus concerning the order of the two incisions. Although there is agreement that the 5' and 3' incisions are made in a near-synchronous manner (Moggs et al., 1996), both 5' uncoupled (Matsunaga et al., 1995; Moggs et al., 1996) and 3' uncoupled (Evans et al.,

1997a; Evans et al., 1997b; Mu et al., 1996) incisions have been observed in different experimental contexts *in vitro*. Using catalytically inactive forms of XPG it has been shown that the presence of XPG, but not its catalytic activity, is required for the generation of the 5' incision by XPF-ERCC1 (Constantinou et al., 1999; Wakasugi et al., 1997). Another study showed that the efficient 3' incision by XPG required the presence and catalytic activity of XPF-ERCC1 (Tapias et al., 2004).

Here we report the use of catalytically inactive mutants of XPF and XPG to establish the relative temporal order of the two incision reactions and DNA repair synthesis in human cell-free extracts and cells. The results provide a new model for a mechanism whereby the dual incision and repair synthesis events of NER are highly coordinated. In turn, the model can explain how the potentially dangerous effects of ssDNA intermediates are minimized or even prevented.

RESULTS

Active Site Mutants of XPG and XPF do not Support Dual Incision

Because the dual incision and repair synthesis steps in NER occur in a near simultaneous manner, it has not been unequivocally established whether there is a temporal order to the 5' and 3' incisions. We reasoned that a systematic investigation using mutants of XPF-ERCC1 and XPG that are catalytically inactive, but retain full DNA binding ability might be suitable to trap NER intermediates and to determine the relative order of the dual incision and

repair synthesis steps. Three useful active site mutants of XPG have been reported. These D77A, E791A and D812A proteins do not display nuclease activity and prevent dual incision in NER *in vitro*, but allow 5' incision by XPF-ERCC1 to occur (Constantinou et al., 1999; Wakasugi et al., 1997). We have recently characterized the active site of human XPF and constructed several mutants with severely impaired endonuclease activity that retain full DNA binding activity (Enzlin and Schärer, 2002). Here, we further characterized these mutants to select a catalytically active mutant devoid of NER dual incision activity analogous to the previously characterized XPG mutants. Hence, we tested the ability of these purified recombinant proteins, expressed as heterodimers with ERCC1, to restore NER activity in XP-F deficient cell extracts (Moggs et al., 1996). The wild-type, R678A, R681A, E701A and R715A XPF proteins fully restored the excision of a lesion-oligonucleotide from a plasmid containing a site-specific 1,3-intrastrand d(GpTpG) cisplatin DNA crosslink (Figure 1, lanes 1, 3, 5, 6 and 9, respectively), whereas E679A displayed residual activity (Figure 1, lane 4). By contrast, the D676A, D704A, E714A, K716A and D720A XPF mutants (Figure 1, lanes 2, 7, 8, 10 and 11, respectively) failed to restore any NER activity. An earlier study using a fully reconstituted system showed that XPF-D676A was devoid of any NER activity, whereas D720A had some residual activity (Tapias et al., 2004).

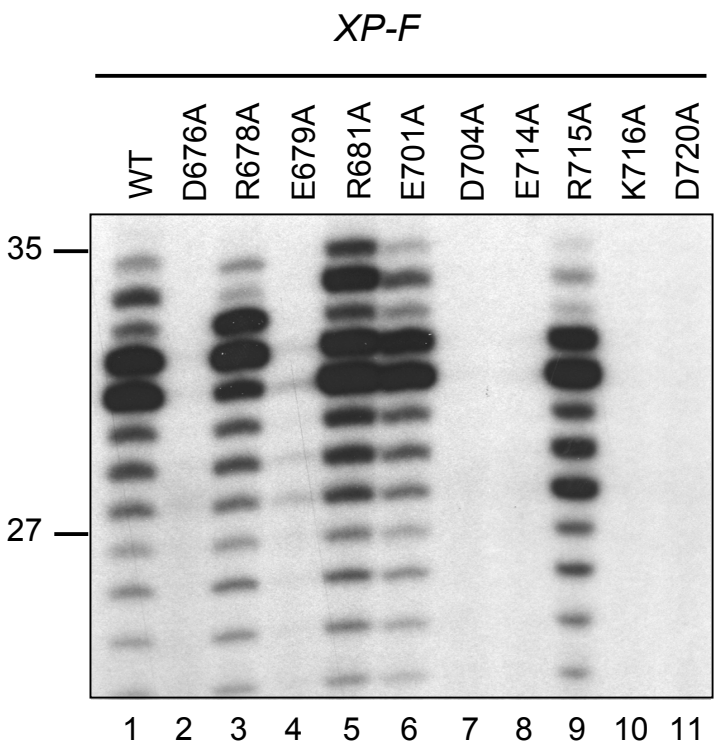


Fig. 1. XPF Active Site Mutants Deficient in NER *in vitro.* Cell extracts prepared from XPF-deficient XP2YO cells were incubated with a plasmid containing a single 1,3 cisplatin intrastrand crosslink in the presence of 200 fmol purified recombinant wild-type XPF-ERCC1 (lane 1) or XPF-ERCC1 proteins with different point mutations in XPF (lanes 2-11). The excision products containing the cisplatin adduct were labeled by the annealing of an oligonucleotide complementary to the excised oligonucleotides with a G_4 overhang and filling in with Sequenase 2.0 and [α-^{32}P] dCTP. The products were separated on a 14% denaturing polyacrylamide gel and visualized by autoradiography. The positions of size markers are indicated on the left.

Efficient 3' Incision by XPG is Dependent on Prior 5' Incision by XPF-ERCC1

We used XPF-ERCC1-D676A and XPG-E791A for further studies to discern any possible interdependency of the 5' and 3' incision steps. Covalently closed circular DNA containing a single 1,3-intrastrand cisplatin DNA crosslink was incubated with an XPG- or XPF-deficient cell-free extract complemented with wild-type or nuclease-deficient XPG and XPF proteins. The reaction products were purified and cleaved with *Bss*HII to excise a 190 bp fragment from

the plasmid DNA. Incision products were detected in two ways: *(i)* filling in the 3'-recessed ends formed by *Bss*HII cleavage to generate a 130 nt fragment for the 5' uncoupled incision by XPF and a 100 nt fragment for incision by XPG, in the presence or absence of the 5' incision (Figure 2A). *(ii)* Annealing of the product to an oligonucleotide complementary to the excised fragment with a G_4 overhang followed by a fill-in reaction would yield a 90mer product for an uncoupled 3' incision XPG and a product of about 30 nt for dual incision.

Incubation of the plasmid with XP-G or XP-F cell extract did not yield any incision products (Figure 2B, lanes 2 and 5, respectively). When the extracts were complemented with wild-type XPG or XPF proteins (lanes 3 and 6, respectively), products specific for dual incision (25-33 nt in length) as well as the product of XPG cleavage (around 100 nt) were visible. We have consistently observed a difference in the amount of dual incision product in complemented XP-G and XP-F cell extracts (compare lanes 3 and 6), which we attribute to a difference of NER proficiency of the two cell extracts. Addition of XPF-D676A to the XP-F cell extract did not result in any additional products compared to XP-F extract alone (compare lanes 5 and 7), suggesting that 3' uncoupled incision by XPG was not detectable in the presence of catalytically inactive XPF. By contrast, addition of XPG-E791A to the XP-G cell extract resulted in the appearance of a specific band (of around 130 nt in length) corresponding to the product of XPF 5'-uncoupled incision (lane 4). No 3'-uncoupled incisions were observed under the conditions used in our assays. These results indicate that 3'

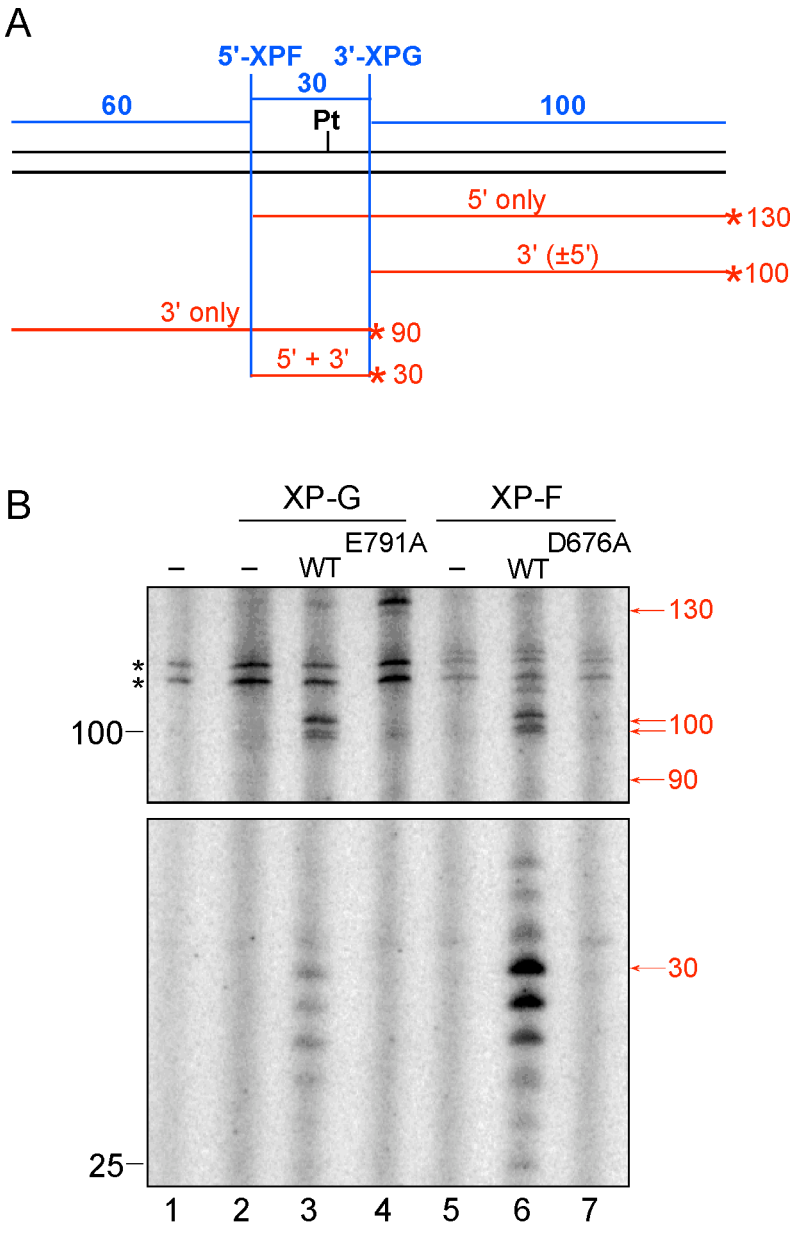


Fig. 2. XPG Cleavage is Dependent on XPF-ERCC1 Cleavage. (A) Schematic representation of a 190bp *Bss*HII fragment with a single defined cisplatin lesion. Incision sites by XPF-ERCC1 and XPG are indicated. Incisions were detected using fill-in reactions with Sequenase 2.0 and [α-^{32}P] dCTP by annealing an oligonucleotide complementary to the excision product containing a G_4 overhang (for the 90 and 30mers) as well as by direct labeling of DNA at the sites of *Bss*HII cleavage (for the 130 and 100mers). All the possible excision products are indicated in red. The position of the [α-^{32}P] label is indicated by an asterisk. **(B)** cccDNA with a single defined cisplatin lesion was incubated with no (lane 1), XP-G (lanes 2-4) or XP-F (lanes 5-7) cell extract, either alone (lanes 2 and 5) or complemented with wild-type XPG (lane 3), XPG-E791A (lane 4), wild-type XPF (lane 6), XPF-D676A (lane 7), purified, digested with *Bss*HII, radioactively labeled and analyzed on a denaturing PAGE gel. The positions of size markers are indicated on the left, the position of the expected reaction products on the right of the gel. Two unspecific bands present in all the lanes are marked by asterisk.

incision by XPG is dependent on the presence and catalytic activity of XPF-ERCC1, while the presence, but not the catalytic activity of XPG is required for the 5' incision by XPF-ERCC1. Hence, the 5' incision might precede the 3' incision.

DNA Repair Synthesis can be Initiated *in vitro* without the 3' Incision by XPG

To determine if both 5' and 3' incisions need to occur before DNA repair synthesis can be initiated, we investigated the nature of the repair synthesis products in XP-G cell extracts complemented with wild-type XPG and XPG-E791A, by incubating a plasmid containing a single defined cisplatin lesion in the extracts together with [α-^{32}P]-dCTP and [α-^{32}P]-TTP. After the reaction, DNA was purified and digested with *Kpn*I and/or *Sac*I or *Xho*I and/or *Bsr*BI (Figure 3A).

When DNA was incubated with the XP-G cell extract alone, products of nonspecific DNA synthesis were observed with signal intensities roughly proportional to the length of the DNA fragments (Figure 3B, lanes 1-6). These signals are likely due to random nicks produced by topoisomerases and/or nucleases present in the cell-free extracts (Hansson et al., 1989). Addition of wild-type XPG to the mixture led to a significant increase in the intensity of the bands of 112 nt (*Kpn*I and *Sac*I) and 79 nt (*Bsr*BI and *Xho*I), corresponding to newly synthesized and ligated DNA at the site of the cisplatin lesion (lanes 9 and 12, respectively). Note that the 79 nt signal of the *Bsr*BI/*Xho*I digestion in lane 12 is more intense than the non-specific signal at 108 nt, strongly suggesting that the 79 bp band results from XPG-induced repair synthesis. Addition of nuclease

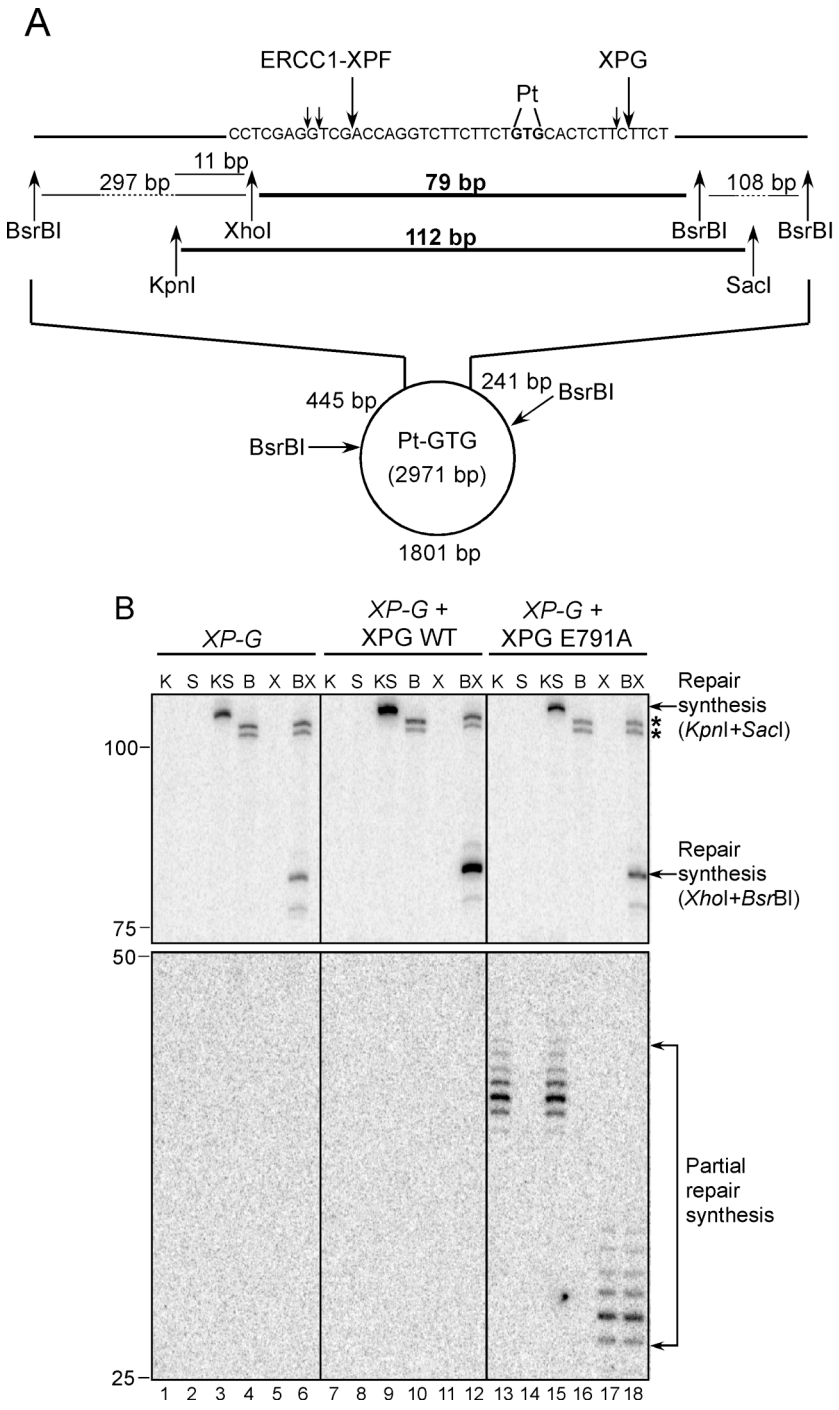


Fig. 3. XPG-E791A Supports Partial DNA Repair Synthesis *in vitro*. (A) Schematic representation of the lesion-containing plasmid used in the assay. Incision sites by XPF-ERCC1 and XPG and the sizes of restriction fragments are indicated. The fragments containing the repair synthesis products are shown in bold (79 bp and 112 bp). **(B)** cccDNA with a single defined cisplatin lesion was incubated with a XP-G cell extract, either alone (lanes 1-6) or in the presence of 600 fmol of wild-type XPG (lanes 7-12) or XPG-E791A (lanes 13-18) as well as 10 µCi of [α-^{32}P]dCTP and 10µCi [α-^{32}P]TTP. DNA was further purified, digested with *Kpn*I (lanes 1, 7, 13), *Sac*I (lanes 2, 8, 14), *Kpn*I+*Sac*I (lanes 3, 9, 15), *Bsr*BI (lanes 4, 10, 16), *Xho*I (lanes 5, 11, 17), *Bsr*BI+*Xho*I (lanes 6, 12, 18) and analyzed on a denaturing PAGE gel. The positions of size markers are indicated on the left, the nature of the observed products on the right of the gel. Two unspecific bands are marked by asterisk. Abbreviations: K= *Kpn*I, S= *Sac*I, KS= *Kpn*I+*Sac*I, B= *Bsr*BI, X= *Xho*I, BX= *Bsr*BI+*Xho*I.

deficient XPG-E791A, which permits incision by XPF-ERCC1, to the XP-G cell extracts, did not result in a change in the intensity of the full-length repair synthesis products of 112 bp and 79 bp. However, two new products with the most intensive bands of 39 nt (*Kpn*I and *Sac*I) and 28 nt (*Bsr*BI and *Xho*I) were visible after a longer exposure of the gel (lanes 15 and 18, respectively). These bands were also present after the cleavage with only one restriction enzyme 5' to the damaged site (lanes 13 and 17), while no products were visible after cutting with restriction enzymes 3' to the incision sites (lanes 14 and 16). The appearance of these bands is therefore consistent with their being partial repair synthesis products, in which the polymerase extended the 3'-OH group generated by XPF-ERCC1 about 18-20 nt in the absence of XPG incision. These observations indicate that initiation of repair synthesis is dependent on cleavage by XPF-ERCC1 and that it can occur prior to 3' cleavage by XPG. No partial repair synthesis products were observed when an extract made from XP-F cells was complemented with wild-type XPF or XPF-D676A (data not shown). These results demonstrate that repair synthesis can be initiated *in vitro* prior to the 3' incision by XPG.

Catalytically Inactive XPF Persists at Sites of UV Damage

Having observed partial repair synthesis without XPG cleavage *in vitro*, we wished to test whether repair synthesis could also be initiated *in vivo* prior to 3' incision by XPG. Therefore we used a XP-G cell line expressing wild-type XPG or XPG-E791A and a XP-F cell line expressing wild-type XPF or XPF-D676A. We

have previously described the generation and characterization of XP-G cell lines expressing wild-type XPG and XPG-E791A using lentiviral transduction. Both XPG proteins were localized to UV-damaged spots in cell nuclei shortly after damage infliction, but only XPG-E791A persisted in these spots, suggesting that completion of NER is required for the dissociation of XPG (Thorel et al., 2004). XP-F cell lines expressing wild-type XPF and XPF-D676A were generated in an analogous fashion by transducing XPF-deficient XP2YO cells with lentiviral recombinants encoding HA-tagged wild-type XPF and XPF-D676A. The resulting cells were subjected to local UV irradiation and the recruitment of XPC, the initial damage recognition factor, and XPF to sites of UV damage was analyzed (Volker et al., 2001). At 0.5 hrs after irradiation, we observed co-localization of XPC with both HA-tagged wild-type XPF and XPF-D676A (Figure 4A). 3 hours after local UV irradiation, XPC and XPF were no longer present at the damaged sites in the cells expressing wild-type XPF. However, in the mutant XPF transductants XPC remained co-localized with the catalytically deficient XPF-D676A (Figure 4B). Similarly, XPG-E791A transduced in XP-G cells persisted at sites of local UV damage, while wild-type XPG did not (Thorel et al., 2004). Hence, cleavage by XPF-ERCC1 and XPG is needed for both nucleases to dissociate from the damaged site and for the completion of NER.

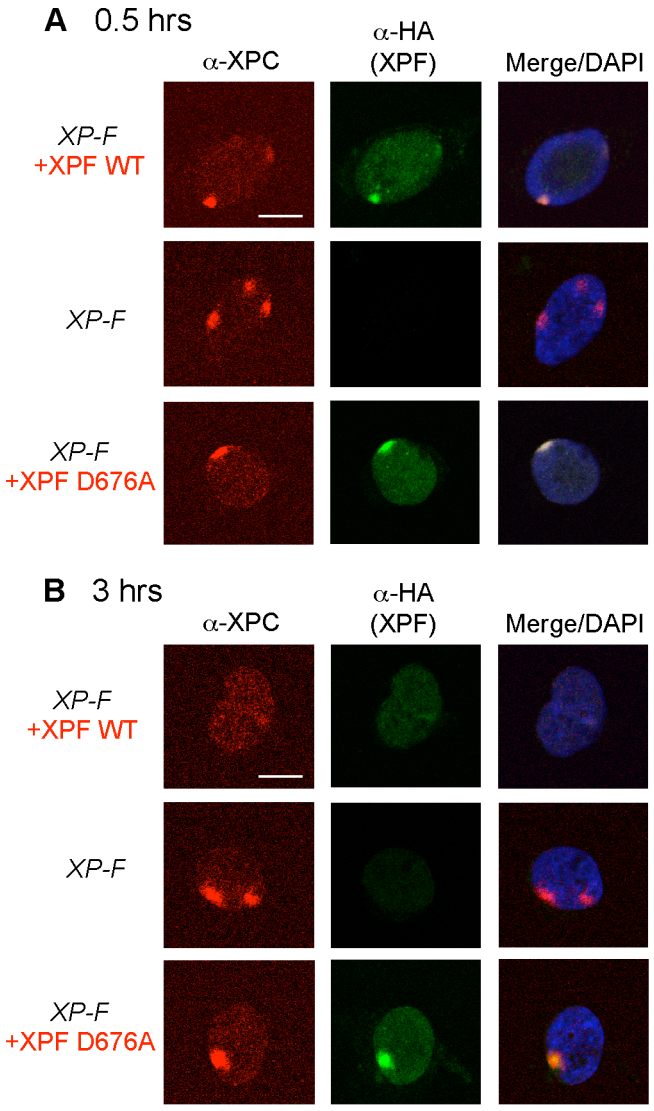
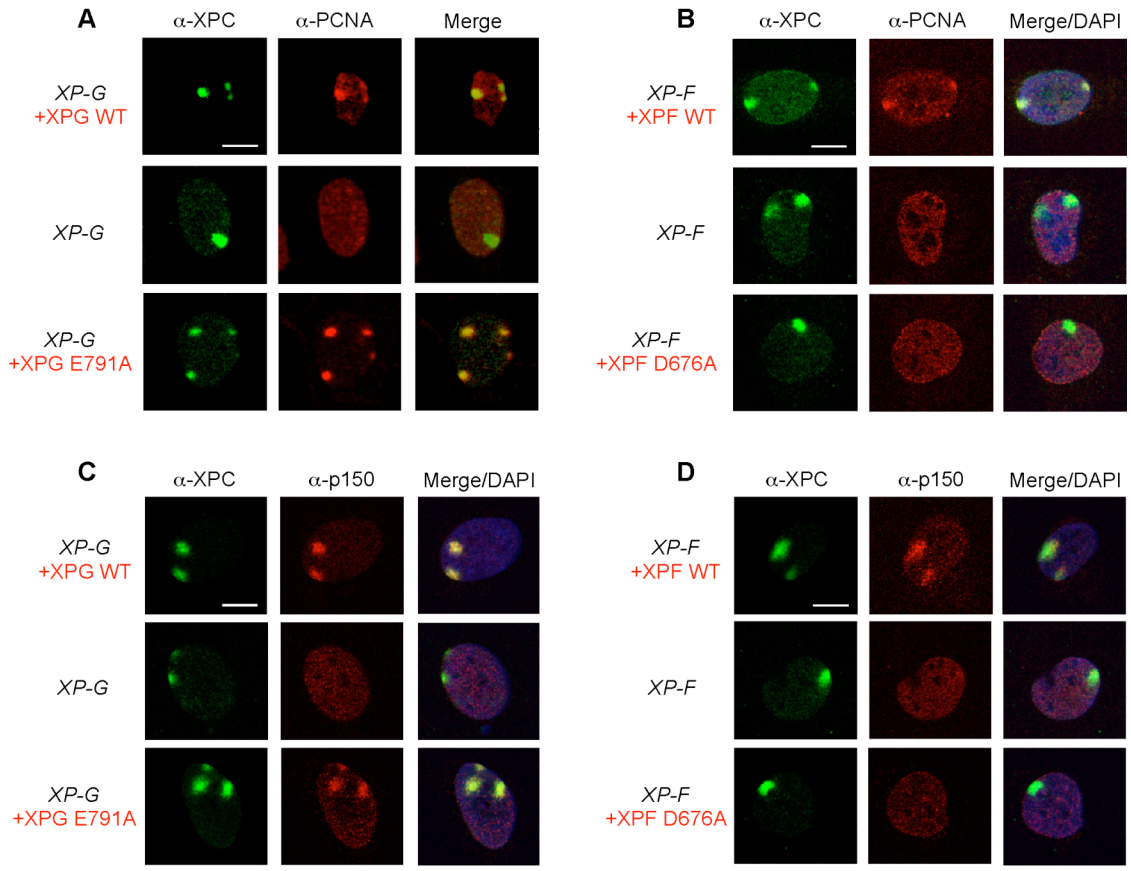


Fig. 4. Recruitment of XPF to Sites of Local UV Damage in Different XP-F Cell Lines. XP2YO (XP-F) cells, untransduced or transduced with XPF-WT or XPF-D676A, were grown on coverslips and locally irradiated with a UV dose of 150 J/m^2 through filters with 5 μm pores and fixed 0.5 hours **(A)** or 3 hours **(B)** after irradiation. The cells were immunolabeled with antibodies against XPC (red) or the HA tag present on the C-terminus of XPF (green). Merged images indicate the overlay of XPC, XPF and DAPI staining. Scale bars, 10 μm.

Recruitment of PCNA and CAF-1 to Sites of Local UV Damage Depends on the Presence, but not the Catalytic Activity of XPG

For repair synthesis to take place, the DNA replication machinery has to be recruited to the sites of DNA damage. Therefore we decided to follow the localization of PCNA, a component of the DNA replication machinery, after local UV irradiation. Using the transduced XP-G and XP-F cell lines, we studied how the catalytic activity of the two nucleases correlates with the recruitment of PCNA to damaged sites. It has been shown before that recruitment of PCNA to the sites of local UV damage is severely affected in XPG-deficient cells (Essers et al., 2005). In agreement with this report, we did not observe any co-localization of PCNA with XPC in XP-G cells 0.5 hrs after UV irradiation (Figure 5A, middle row). However, as expected, XPC and PCNA co-localized at sites of UV damage in XP-G cells expressing wild-type XPG (Figure 5A, top row). PCNA also co-localized with XPC in the XPG-E791A transductants (Figure 5A, bottom row). In principle, the recruitment of PCNA to sites of UV damage in the presence of catalytically inactive XPG could reflect partial DNA repair synthesis or be due to the recruitment of PCNA prior to incision, possibly by direct interaction with XPG. To distinguish between these possibilities, we investigated the recruitment of PCNA in various XP-F cells. While PCNA was found at sites of UV damage in XP-F cells expressing wild-type XPF, PCNA did not colocalize with XPC in untransduced XP-F cells or in the XPF-D676A transductants (Figure 5B). These observations demonstrate that the recruitment of PCNA to sites of UV damage is dependent on the catalytic activity of XPF and therefore likely on active DNA



repair synthesis.

Fig. 5. XPF- and XPG-Dependent Co-localization of PCNA and CAF-1 with XPC. XPCS1RO (XP-G) cells, untransduced or transduced with XPG-WT or XPG-E791A (**A** and **C**) and XP2YO (XP-F) cells, untransduced or transduced with XPF-WT or XPF-D676A (**B** and **D**), were grown on coverslips and locally irradiated with a UV dose of 150 J/m^2 through filters with 5 μm pores and fixed 0.5 hours after irradiation. The cells were immunolabeled with antibodies against XPC (green), PCNA (red) and p150, the largest subunit of CAF-1 (red). Merged images indicate the overlay of XPC, PCNA or p150 and DAPI staining. Scale bars, 10 μm.

Having established that the recruitment of the replication machinery required the catalytic activity of XPF, but not that of XPG, we asked if factors

acting even later in NER could be recruited to sites of UV damage in the absence of XPG incision. We examined the recruitment of p150, a subunit of the chromatin assembly factor 1 (CAF-1) that is involved in the restoration of chromatin after a NER reaction (Green and Almouzni, 2003). p150 behaved like PCNA in that its recruitment was dependent on the catalytic activity of XPF, but not on that of XPG (Figure 5D and 5C). These results are consistent with an earlier observation that CAF-1 is recruited to the sites of DNA damage in a PCNA-dependent manner (Green and Almouzni, 2003). The important novel conclusion is that even factors acting downstream of DNA repair synthesis can be recruited to sites of UV damage before the second incision 3' to the lesion has occurred.

Partial Unscheduled DNA Synthesis (UDS) Occurs in the Absence of 3' Incision by XPG

To test whether loading of PCNA to NER sites is able to stimulate DNA repair synthesis in the absence of 3' incision *in vivo*, we examined DNA repair by unscheduled DNA synthesis (UDS) after UV-irradiation of XP-G transductants expressing wild-type XPG or XPG-E791A. Wild-type XPG complemented the severe UDS defect of untransduced XP-G cells (102 % versus 7%, with NER-proficient cells assayed in parallel set at 100%, Figure 6A). In line with the observed partial DNA repair synthesis *in vitro* and recruitment of PCNA, the very low UDS level of untransduced XP-G cells was significantly increased upon expression of the catalytically inactive XPG (from 7 to 49 % UDS, Figure 6A).

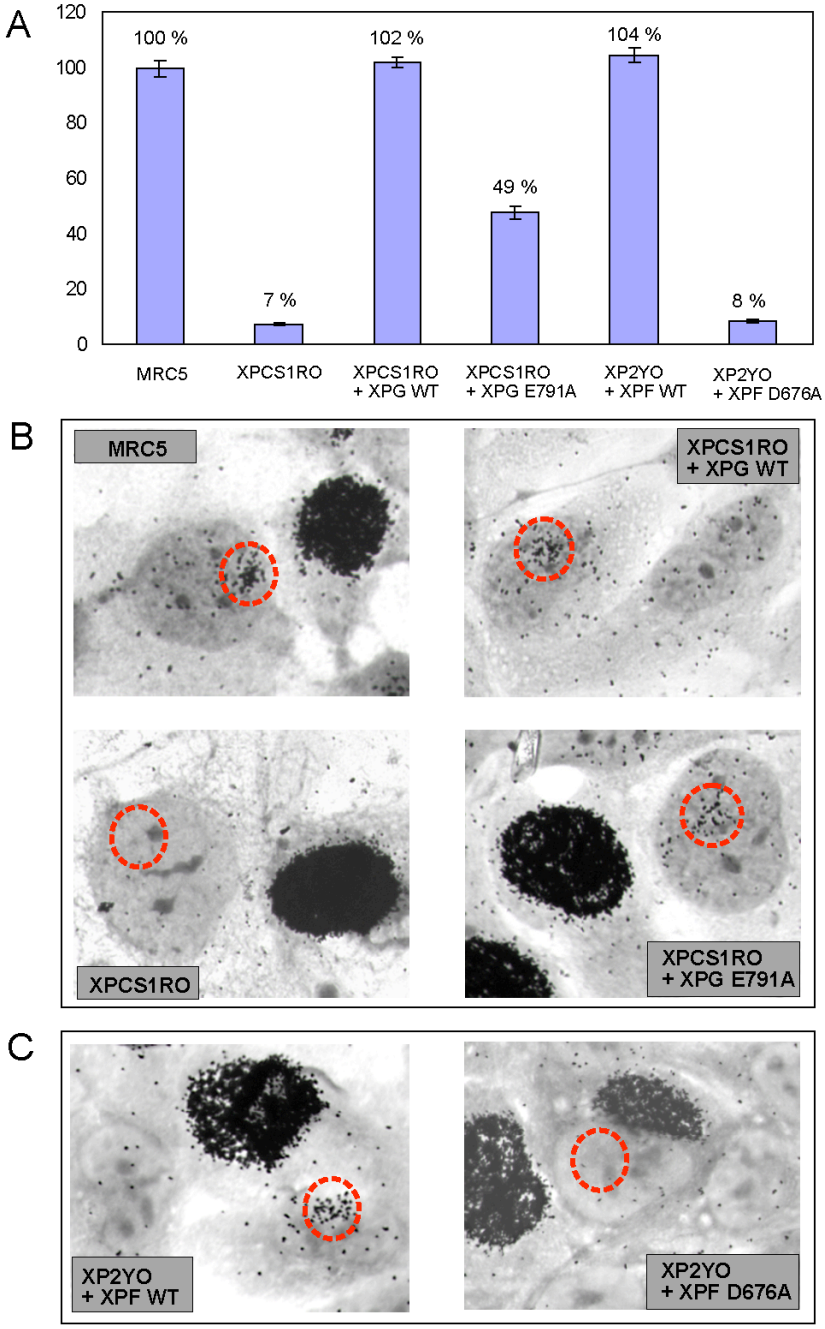


Fig. 6. UDS in XP-F and XP-G Cells Transduced with Wild-Type and Mutant *XPF* and *XPG*, Respectively. (A). DNA repair synthesis or UV-induced UDS levels of different, as indicated, XP-F and XP-G cells, expressed as percentage of the UDS of a NER-proficient cell line (MRC5) assayed in parallel. (**B** and **C**) UDS in cells locally irradiated through a 5 μm microporous filter (60 J/m^2), NER-proficient MRC, XPCS1RO, XPCS1RO transduced with wild type XPG and XPG-E791A (**B**) and XP2YO transduced with wild-type XPF or XPF-D676A (**C**). Heavily labeled cells were cells in S-phase, incorporating large amounts of tritiated thymidine by replicative DNA synthesis. Dotted circles indicate the positions of the pores.

To try to ensure that this UV-induced DNA synthesis occurs at sites of NER rather than being a non-specific artifact, we monitored repair synthesis at locally UV-damaged areas (Figure 6B). Although quantification is difficult, significant numbers of autoradiographic grains were found clustered together over non-S phase nuclei in NER-proficient cells and XP-G transductants expressing wild-type XPG Figure 6B, upper panels). Very few grains were found over comparable nuclear areas of untransduced XP-G cells but the nuclei of the XPG-E791A transductants exhibited a significant amount of grain clustering, roughly mid-way between the wild-type and XP-G levels (Figure 6B, lower panels). In contrast, the levels of UDS in XP-F cells expressing XPF-D676A (8% of wild-type, Figure 6A) was at the same background level as untransduced XP-F cells, whereas UDS was restored to normal levels in cells expressing wild-type XPF (104%, Figure 6A). Local UDS experiments confirmed these findings; while UDS sites were clearly observed for XP-F transductants expressing wild-type XPF, they were not evident in XPF-D676A transductants (Figure 6C). Together, these results strongly suggest that the observed UDS is linked to sites of local UV damage, and that partial repair synthesis can occur in living cells in the absence of the catalytic activity of XPG, whereas it does require the catalytic activity of XPF.

DISCUSSION

Excision of an oligonucleotide containing a lesion in human nucleotide excision repair formally generates a ssDNA gap of about 30 nt in length, another potentially deleterious DNA lesion. It is therefore important that the dual incision and repair synthesis steps are tightly coordinated. One crucial aspect of the coordination of these two steps is that no incisions are made in the DNA before the repair synthesis machinery is in place to initiate repair synthesis. Here we explored the hypothesis that a coordination of the two incision steps, 5' and 3' to the lesion by XPF-ERCC1 and XPG, respectively, with the initiation of repair synthesis ensures that a ssDNA gap is not exposed in the process.

A Defined Order of Incisions and Repair Synthesis in NER?

One way of coordinating the dual incision and repair synthesis steps and minimizing the time in which a ssDNA gap may be exposed would be to make the incision 5' to the lesion by XPF-ERCC1 first, use the free 3'-OH group generated in this way to initiate repair synthesis and only perform 3' incision by XPG once repair synthesis is underway. Since the two incision reactions occur in near simultaneous fashion (Moggs et al., 1996; Riedl et al., 2003), we have examined this possibility by using catalytically inactive XPF-ERCC1 and XPG proteins with the hope of trapping NER intermediates.

We believe this approach has been useful and present several lines of evidence supporting the model outlined above: First, we found the 5' incision by

XPF-ERCC1 to be dependent on the presence, but not catalytic activity of XPG, while the 3' incision by XPG was dependent on the catalytic activity of XPF-ERCC1 (Figure 2), extending previous findings (Constantinou et al., 1999; Tapias et al., 2004; Wakasugi et al., 1997). Second, we have observed products of partial DNA synthesis *in vitro* in the presence of catalytically inactive XPG (Figure 3), demonstrating that the incision 5' to the lesion by XPF-ERCC1 is necessary and sufficient for the initiation of repair synthesis, while 3' incision by XPG is needed for the completion, but not the initiation of repair synthesis. Third, we found that some late NER factors, including the replication factor PCNA and chromatin assembly factor CAF-1, are recruited to the sites of local UV damage in cells expressing catalytically inactive XPG, but not in cells expressing catalytically inactive XPF (Figure 5). Fourth, cells expressing catalytically inactive XPG, but not those expressing catalytically inactive XPF, are capable of undergoing intermediate levels of unscheduled DNA repair synthesis (Figure 6).

Based on these observations we suggest that the human NER pathway has a defined temporal order for the 5' and 3' incisions and for DNA repair synthesis. Specifically, we propose the following model for the coordination of the dual incision and repair synthesis steps (Figure 7). After assembly of all the factors of the preincision complex, 5' cleavage by XPF-ERCC1 takes place, generating a free 3'-OH group. The repair synthesis machinery consisting minimally of polymerase δ, the clamp loader RFC and the processivity factor PCNA are recruited and repair synthesis is initiated. Which factors and

interactions may facilitate this recruitment remains to be established, but it is possible that RPA has an important role in this transition (Riedl et al., 2003).

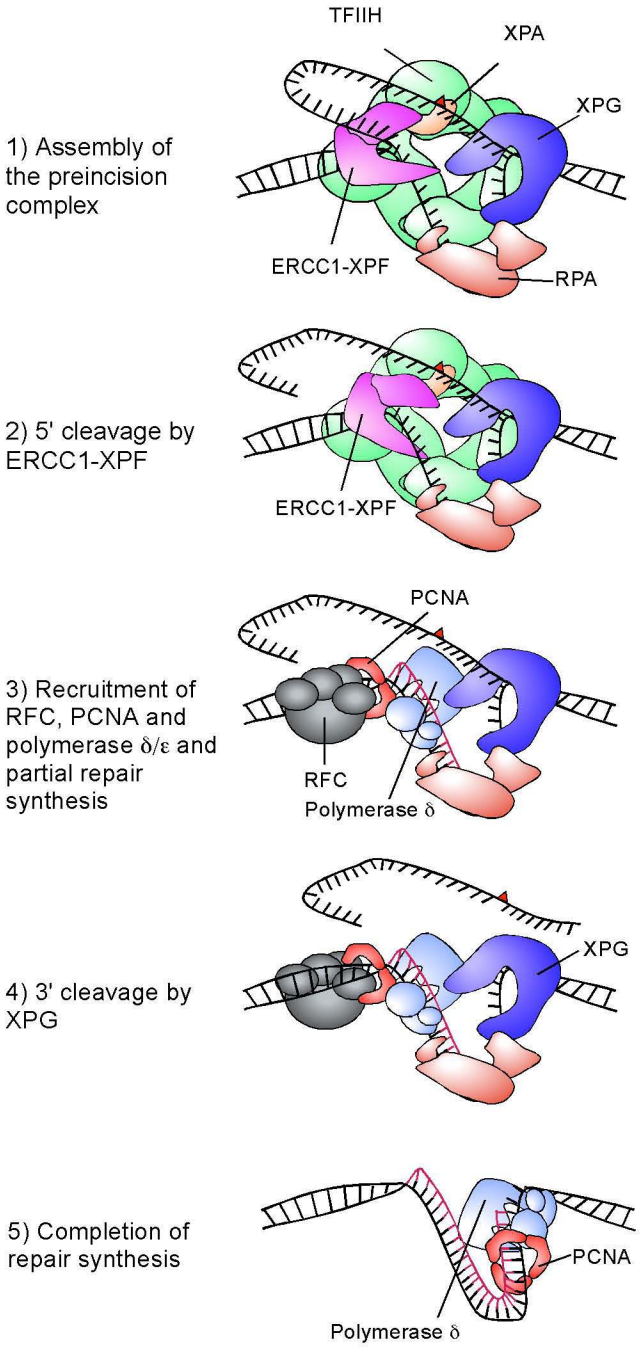


Fig. 7. Model for the Coordination of Dual Incision and Repair Synthesis Steps in NER. Schematic representation of the proposed sequence of events following the assembly of the preincision complex. The red triangle stands for the DNA lesion. Individual proteins involved in each step are indicated.

DNA synthesis is then initiated and proceeds about two-thirds through the repair patch. The stalling of the polymerase at this point activates XPG endonuclease activity and repair synthesis is completed. We have previously shown that XPG has distinct requirements for binding and cleaving DNA (Hohl et al., 2003) raising the possibility that a conformational change in the NER complex brought about by the polymerase activity triggers the catalytic activity of XPG.

The model proposed here does not exclude an involvement of additional protein-protein interactions or protein modifications in the various steps. For example, it is known that XPG has a PIP box and that it can interact with PCNA (Gary et al., 1997). Although it has not yet been shown convincingly that the interaction between PCNA and XPG is required for NER, an interaction between the two proteins may contribute to the activation of the XPG incision. A recent study has implicated polκ in the repair synthesis step of NER (Ogi and Lehmann, 2006). It is possible that two polymerases, polδ and polκ, act at different steps of repair synthesis, for example before and after incision by XPG has taken place. Further studies will be required to determine how the various phases of repair synthesis in NER are regulated by protein-protein interactions and post-translational modifications.

"Cut-and-Patch" or "Patch-and-Cut"?

Early investigations of excision repair in bacteria focused on the discrimination between the two models: "patch and cut", involving a first incision close to the damage, followed by repair synthesis and second incision, and "cut and patch", invoking excision of the damaged base/s prior to repair synthesis

(Kondo, 1974). The “patch and cut” mechanism was also consistent with observations in mammalian cells showing that the kinetics of DNA incision and repair synthesis were faster than the disappearance of pyrimidine dimers (Ehmann and Friedberg, 1980; Williams and Cleaver, 1978). Subsequently however, in particular with the ability to reconstitute NER *in vitro* and the ability to observe dual incision on NER substrates in the absence of repair synthesis, the “cut and patch” model has been accepted as the way by which NER operates (Aboussekhra et al., 1995; Araujo et al., 2000; Moggs et al., 1996; Mu et al., 1995). Our present work suggests that the human NER machinery operates via a “cut-patch-cut-patch” mechanism that includes features of both previous models. Interestingly, long-patch base excision repair (BER) has been shown to proceed in a similar way. In long-patch BER, polymerase δ/ε, supported by the replication accessory factors RFC and PCNA or polymerase β, carries out repair synthesis past the abasic site and introduces 2-6 nucleotides. The short oligonucleotide overhang generated in this way is excised by the Flap endonuclease FEN-1, and the nick is sealed by DNA ligase I (Matsumoto et al., 1999; Pascucci et al., 1999). In line with this model, it was demonstrated that PCNA facilitates excision in long-patch BER (Gary et al., 1999). Stimulation of the dual incision by PCNA has also been observed in NER, leading to the proposal that PCNA may promote the turnover of the early NER factors (Nichols and Sancar, 1992) linking the excision and repair synthesis steps.

Why Are Uncoupled 3' Incisions by XPG Sometimes Observed?

One set of experimental observations appears to be in contrast with our results. Under very similar experimental conditions either 5' uncoupled (Matsunaga et al., 1995; Moggs et al., 1996) or 3' uncoupled incisions (Evans et al., 1997a; Evans et al., 1997b; Matsunaga et al., 1995; Mu et al., 1996) have been observed. Our model would predict that 3' uncoupled incision by XPG should not occur at any appreciable frequency. One possible explanation is that XPF-ERCC1 and XPG, as structure-specific endonucleases with an ability to cleave different model substrates *in vitro* without the need for additional proteins (O'Donovan et al., 1994; Sijbers et al., 1996), can incise NER intermediates under certain conditions *in vitro* that would be disfavored *in vivo*. For example, high relative concentration of XPG or the absence of XPF-ERCC1 in a cell extract or reconstituted system may allow incision by XPG 3' to the lesion in the absence of a properly assembled NER complex. We have previously shown that XPG has distinct requirements for binding and cleaving DNA substrates and that the XPG spacer region has a critical role in mediating this substrate preference (Dunand-Sauthier et al., 2005; Hohl et al., 2007; Hohl et al., 2003). We suggest that XPG is present in a catalytically inactive conformation prior to 5' incision and partial repair synthesis and that its catalytic activity is revealed by a change in the complex brought about by partial repair synthesis. The barrier for XPG to cleave certain substrates does not occur at the level of substrate binding, but likely involves a subsequent rearrangement of an XPG-substrate complex (Hohl et al.,

2003). We propose that the observed uncoupled 3' incision could be due to a lowering of this activation barrier under certain experimental conditions, leading to 3' uncoupled incision.

Our model requires further investigations of the biochemical nature of the intermediates generated during the 5' incision, repair synthesis and 3' incision reactions. It also remains to be seen to what extent post-translational modifications or additional protein factors contribute to the regulation of the dual incision and repair synthesis steps. We believe that the data and model presented here provide a framework for further studies to understand the tight coordination of various steps in the NER pathway to avoid the formation of potentially very dangerous reaction byproducts such as ssDNA gaps and nicks.

EXPERIMENTAL PROCEDURES

Protein Purification

Wild type XPF, XPF D676A, XPF R678A, XPF E679A, XPF 681A, XPF E701A, XPF D704A, XPF E714A, XPF R715A, XPF K716A, XPF D720A, wild type XPG and XPG E791A proteins were expressed in Sf9 insect cells and purified as described previously (Enzlin and Schärer, 2002; Hohl et al., 2003). Typically 0.2-0.5 mg of proteins were obtained at concentrations of 0.2-0.3 mg/ml.

In vitro NER Dual Incision Assay

Covalently closed circular DNA (pBluescript II SK(+)) containing a single 1,3-intrastrand d(GpTpG) cisplatin-DNA crosslink was prepared as described previously (Moggs et al., 1996) and additionally purified over two consecutive sucrose gradients. Reactions were carried out in a buffer containing 40 mM HEPES-KOH (pH 7.8), 70 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP, 0.36 mg/ml BSA, 22 mM phosphocreatine (di-Tris salt) and 50 ng/μl creatine phosphokinase. Each reaction contained 200 ng DNA and 30 μg of cell-free extract prepared from XPG- or XPF-deficient fibroblast cells (XPCS1RO and XP2YO, respectively). Complementation was assayed upon addition of 730 fmol wild-type or mutant protein (XPG or XPF). Reactions were incubated at 30°C for 45 minutes. DNA was purified using QIAquick Nucleotide Removal Kit (Qiagen) and cleaved with *Bss*HII. 50 nM of an oligonucleotide complementary to the excision product with a G_4 5'-overhang (5'-GGGGGAAGAGTGCACAGAAGAAGACCTGGTCGACC) was added, followed by heat inactivation at 95°C for 5 minutes. After cooling down the reactions for 15 minutes at room temperature, 0.5 units of Sequenase and 3.5 μCi of [α-^{32}P]-dCTP (both from Amersham-Pharmacia, diluted in Sequenase dilution buffer) were added. Reactions were incubated for 3 minutes at 37°C prior the addition of 1.2 μl dNTP mix (100 μM of each dATP, dGTP, TTP and 50 μM dCTP) and incubation for another 12 minutes at 37°C. This fill-in reaction labeled both the *Bss*HII overhangs and the G_4 overhang provided by the complementary oligonucleotide. Reactions were stopped by addition of formamide loading buffer,

heated at 95°C for 5 minutes and analyzed on a 12% denaturing polyacrylamide sequencing gel. The gel was exposed on a phosphor screen and scanned on the PhosphorImager.

In vitro NER Repair Synthesis Assay

The assay was performed using the same substrate, cell extracts and proteins as described for the excision assay. The reaction mixtures additionally contained 10 μM dATP, 10 μM dGTP, 5 μM dCTP, 5 μM TTP, 10 μCi of [α-^{32}P]-dCTP and 10μCi [α-^{32}P]-TTP. Complementation was assayed upon addition of 600 fmol of wild-type or mutant protein (XPG or XPF). Reactions were incubated at 30°C for 3 hours. DNA was purified using MinElute PCR Purification Kit (Qiagen), cleaved with *Kpn*I and *Sac*I or *Bsr*BI and *Xho*I and analyzed on a 10% denaturing polyacrylamide sequencing gel.

Cell Culture Conditions and Preparation of Whole Cell Extracts

For the generation of whole cell extracts, SV40-transformed fibroblast cells XPCS1RO (XPG-deficient, (Ellison et al., 1998) and XP2YO (XPF-deficient, GM08437) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C in the presence of 5% CO2. Cells were grown to near confluency, and whole cell extract was prepared accordingly to a published procedure (Biggerstaff and Wood, 1999).

Cell Transduction with Lentiviral Recombinants

XPG wild type cDNA, XPG-E791A cDNA were cloned into the pLOX/EWGFP lentiviral vector and XPF wild type (with a C-terminal HA tag) and XPF-D676A (with a C-terminal HA tag) cDNAs were cloned into the pWPXL lentiviral vector by replacing the GFP cDNA. Lentiviruses containing the different constructs under the control of the EF1α promoter were produced by co-transfecting 293T cells with the following 3 plasmids: the packaging plasmid pMD2G, the envelop plasmid psPAX2 and the lentiviral vector containing the different XPG and XPF cDNAs. Details of the vectors and protocols are described on the following web site http://www.lentiweb.com. XP-G/CS (94RD27, patient XPCS1RO) and XP-F (XP2YO) SV40 immortalized fibroblasts at 50% confluency were infected with viral particles containing the different XPG and XPF recombinants. Transduced cells were then cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a 5% CO_2 humidified incubator. The transduction efficiency was further assessed by immunofluorescence.

Local UV Irradiation and Immunofluorescence

Local DNA damage infliction within cultured cells was performed as described previously (Mone et al., 2001). Briefly, cells cultured on coverslips were rinsed with PBS and covered with a micro-porous polycarbonate filter containing 5μm pores (Millipore). Cells were irradiated through the filter with a Philips TUV lamp (254 nm) with a dose of 150 J/m^2. After UV-irradiation cells were cultured for 0.5 hrs, washed first with PBS and then with PBS containing

0.05% Triton X-100 for 30 seconds prior to fixation with 3% paraformaldehyde for 15 minutes at room temperature or with ice-cold methanol for 20 min (for PCNA staining). Subsequently, cells were permeabilized by a 2 times 10 min. incubation in PBS containing 0.1% Triton X-100, and washed with PBS^+ (PBS containing 0.15% glycine and 0.5% bovine serum albumin). Cells were incubated at room temperature with the primary antibody (diluted in PBS^+) for 2 hours in a moist chamber. Subsequently cells were washed 5 times for 10 min. with PBS Triton X-100, washed with PBS^+, and incubated at room temperature with the secondary antibody (diluted in PBS^+) for 1 hour in a moist chamber. Cells were washed 5 times for 10 mins in PBS Triton X-100, washed in PBS, and embedded in Vectashield mounting medium (Vector) containing 0.1 mg/ml of DAPI (4′-6′-diamidino-2- phenylindole).

Primary antibodies were as follows: mouse monoclonal anti-PCNA (Dako, clone PC10), 1:1000, rabbit polyclonal affinity purified anti-XPC (Ng et al., 2003), 1:300, mouse monoclonal anti-p150 (CAF-1) (abcam, ab7655), 1:2000, and mouse monoclonal FITC-conjugated anti-HA (Roche, clone 3F10) Secondary antibodies were as follows: Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories), 1:1000, Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories), 1:1000, and Alexa-488 conjugated goat anti-rabbit (Molecular Probes), 1:800.

Confocal Microscopy

Confocal images of the cells were obtained using a Zeiss LSM 510 microscope equipped with a 25mW Ar laser at 488 nm, a He/Ne 543 nm laser,

and a 40 X 1.3 NA oil immersion lens. Alexa-488 was detected using a dichroic beam splitter (HFT 488), and an additional 505- to 530-nm bandpass emission filter. Cy3 was detected using a dichroic beam splitter (HFT 488/543) and a 560- to 615-nm bandpass emission filter.

Unscheduled DNA Synthesis (UDS)

To determine global genome NER activity in cultured cells, UV-induced DNA repair synthesis or unscheduled DNA synthesis (UDS) was measured. Coverslip cultures were rinsed with PBS, UV-irradiated (16 J/m^2, Philips 254 nm TUV lamp) and subsequently incubated for 2 hours in culture medium supplemented with 20μCi/ml [^{3}H-1',2']-thymidine (120 Ci/mmol, Amersham TRK565). After fixation coverslips were dipped in Ilford K2 photographic emulsion, exposed for three days and after development stained with Giemsa. Autoradiographic grains above the nuclei of 50 cells were counted and compared to the number of grains above nuclei of NER-proficient fibroblasts (MRC5, set at 100% UDS), assayed in parallel. UDS in locally damaged cells (local UDS) with 60 J/m^2 was performed in a similar fashion with the exception of an extended exposure time to six days.

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Chapter 3

Influence of RPA, XPA and PCNA on the Catalytic Activity of XPF-ERCC1 and XPG

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SUMMARY

The proper assembly of over 30 proteins involved in nucleotide excision repair (NER) pathway is dependent on numerous specific protein-protein interactions. Proteins involved in early steps recruit proteins acting in later steps. Protein-protein interactions are also responsible for the stability of protein complexes and the activity of some NER proteins. We asked whether early or late NER proteins could modulate the activity of the two endonucleases, XPF-ERCC1 and XPG and found that the 5' incision activity of XPF-ERCC1 on model bubble substrates is stimulated by RPA, but is not influenced by XPA and presence of the DNA damage in one of the strands. The 3' incision activity by XPG is strongly stimulated by PCNA on certain substrates such as a 5' flap substrate and this stimulation is dependent on two Phe residues in the PIP-box of PCNA. However, mutation of these two Phe residues to Ala does not prevent dual incision and repair synthesis *in vitro*.

INTRODUCTION

Nucleotide excision repair (NER) is a complex repair pathway in which over 30 polypeptides collaborate in the excision of damaged sites in DNA in the form of oligonucleotides of 24-32 nucleotides in length. The sequence of events in NER is controlled by a number of established protein-protein interactions (Araujo and Wood, 1999). The interaction between XPC-hHR23B and TFIIH results in recruitment of TFIIH to lesions (Drapkin et al., 1994; Volker et al., 2001; Yokoi et al., 2000). TFIIH is in turn responsible for recruitment of XPG to NER complexes (Dunand-Sauthier et al., 2005; Thorel et al., 2004; Volker et al., 2001). XPF-ERCC1 is recruited through a direct protein-protein interaction between XPA and ERCC1 (Li et al., 1995).

Interactions between NER proteins are not only needed for their recruitment to the damaged sites. It has been shown that RPA stimulates XPF-ERCC1 (Bessho et al., 1997; Matsunaga et al., 1996) and XPG (Matsunaga et al., 1996) incision activity on bubble substrates. However, in another study inhibition of XPG cleavage on a bubble substrate by RPA was observed (Evans et al., 1997). A detailed study analyzed the effect of RPA on the cleavage by the two endonucleases using model substrates containing dsDNA and either 3' or 5' ssDNA protrusions (de Laat et al., 1998). The authors concluded that the interaction between RPA and XPF-ERCC1 or XPG is dependent on the orientation of RPA with regard to the dsDNA-ssDNA junction. If RPA is

positioned on 3' protruding substrates, it has minor inhibitory effect on XPG incision, but strongly stimulates XPF-ERCC1 incision. On the contrary, on 5' protruding substrates it inhibits both incisions.

Another interaction, the function of which is still unclear, is the one between XPG and PCNA (Gary et al., 1997). It was first observed that PCNA physically interacts with and stimulates the structure-specific nuclease activity of an enzyme related to XPG, Fen-1 (DNase IV) (Wu et al., 1996). The PCNA interaction domain in Fen-1 was mapped to a region comprising the residues 328-355. An analogous region was subsequently identified in XPG (residues 981-1009) (Gary et al., 1997). There are several conserved residues in that region, which was later named PIP (PCNA-interacting peptide) box. The core of the PIP box in Fen-1 consists of residues ^{337}QGRLDDFFK^{345} and in XPG of residues ^{990}QLRIDSFFR^{998}. A conserved arginine (Arg^{339} in Fen-1 and Arg^{992} in XPG) was found to be crucial for PCNA binding activity (Gary et al., 1997). Subsequently Leu^{340}, Asp^{341}, Phe^{343} and Phe^{344} in Fen-1 were identified as critical for the interaction between Fen-1 and PCNA, but point mutations of those residues did not abolish PCNA-mediated stimulation of Fen-1 (Frank et al., 2001). Despite the apparent interaction between PCNA and XPG, PCNA-stimulated cleavage by XPG has not yet been observed (Evans et al., 1997). However, stimulation of the excision step in NER by PCNA was reported (Nichols and Sancar, 1992) as well as stimulation of the excision in the long-patch BER by PCNA, which was abolished in a Fen-1 F343A/F344A mutant that does not bind PCNA (Gary et al., 1999).

To characterize the role of PCNA in the excision step of NER, we tested the influence of PCNA on the incision activity of the wild type and F996A/F997A (FF-AA) XPG mutant proteins. We observed a stimulation of the activity of wild type XPG by PCNA, which was abolished in the FF-AA mutant. The mutation did not have a significant effect on dual incision and repair synthesis steps *in vitro*. We have additionally observed stimulation of XPF-ERCC1 cleavage by RPA on bubble substrates, while neither XPA nor the presence of a damaged base in one of the strands had any influence on XPF-ERCC1 incisions.

RESULTS

RPA Stimulates the Activity of XPF-ERCC1 on a Bubble Substrate

It has been shown that RPA stimulates the incision activity of XPF-ERCC1 on model substrates in which a 30 nt long single-stranded overhang is present with the polarity required for incision in NER. We hypothesized that this stimulation should be even more powerful in the presence of XPA and a damaged site in a bubble substrate. This experimental setup is based on the hypothesis that XPA and RPA play a central role in positioning the two endonucleases for the incision on the strand containing the lesion (de Laat et al., 1998; Missura et al., 2001).

To verify this concept, we synthesized a 90mer substrate containing a bubble of 30 nucleotides flanked by two regions of dsDNA. A control bubble

substrate did not contain any modified bases, while a “damaged bubble” substrate contained a dG-AF substrate at position 50 (distance to the ss/dsDNA junction corresponding to the incision site on a dG-AF substrate by the NER machinery in the complete NER reaction) (Gillet et al., 2005).

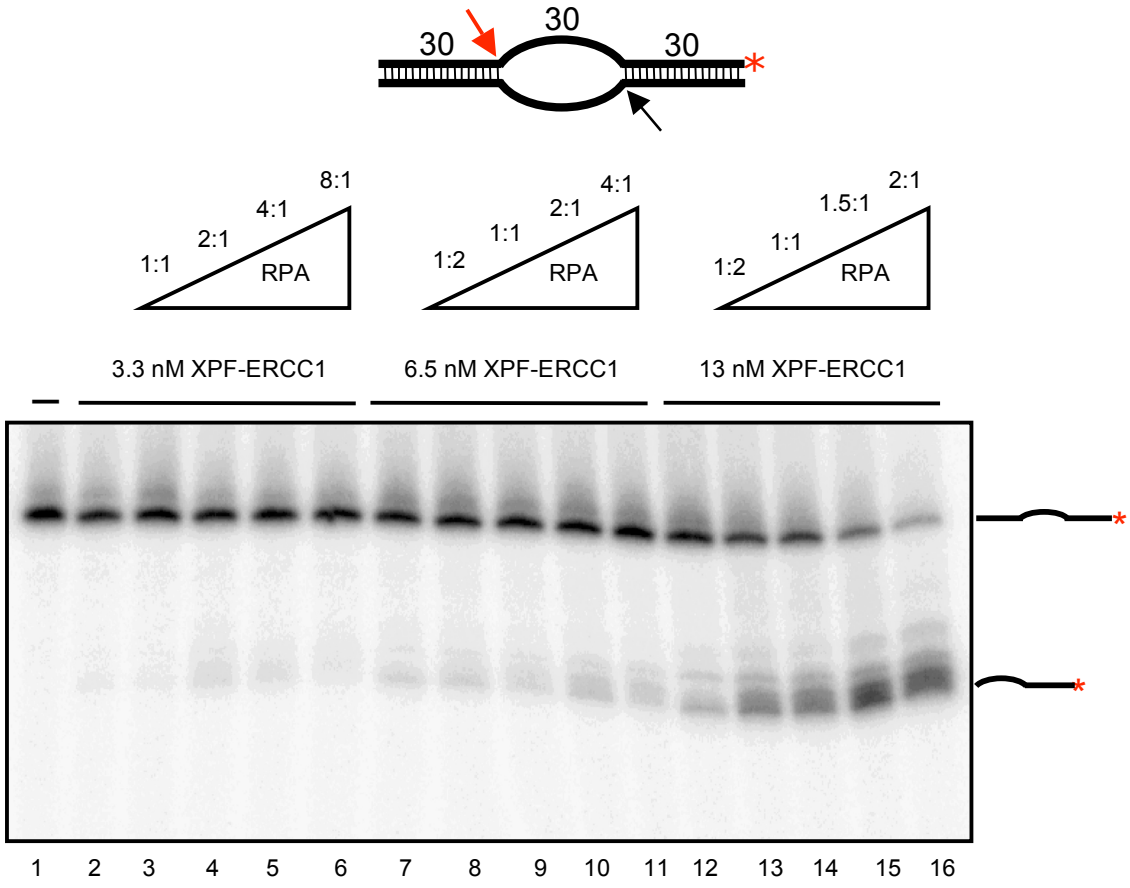

Fig.1. RPA Stimulates Endonuclease Activity of XPF-ERCC1 on a Bubble Substrate. 6.7 nM of a bubble substrate was incubated at 37°C for 45 minutes in presence of 2 mM $MgCl_2$ with wild-type XPF-ERCC1 and RPA. Lane 1, no protein was added. Lanes 2, 7 and 12, only XPF-ERCC1 was added. Lanes 3-6, 8-11 and 13-16, XPF-ERCC1 and RPA were added. The concentrations of XPF-ERCC1 used and the ratio of RPA:XPF-ERCC1 are indicated on the tope of the gel. The position of the 3’ radiolabel on the substrate is indicated by a red asterisk, the positions of the XPF-ERCC1 incisions are marked with arrows, the red one representing the incision site on the labeled strand. The reaction products are shown on the right side of the gel.

First we tested whether we could observe a stimulation of XPF-ERCC1 activity on a bubble substrate without damage, 3'-labeled with ^{32}P, (Figure 1). Using amounts of XPF-ERCC1 which allow only very low incision activity on its own (lanes 2, 7 and 12), we observed a strong stimulation of XPF-ERCC1 incisions by RPA under all the concentrations tested with the most efficient being the molar ratio of RPA to XPF-ERCC1 2:1 (lanes 4, 10 and 16).

When DNA was labeled 5' with ^{32}P on either strand (Figure 2), a ladder of incision products was observed at higher concentrations of XPF-ERCC1 (lanes 7-12, 23-28) as well as with XPF-ERCC1 and RPA together (lanes 13-16, 29-32), with the most prominent band of about 10 nt.

Since such ladder products were not observed in experiments using the 3' labeled substrates, we believe that these additional bands are products of multiple incisions of XPF-ERCC1 on the same substrate. Apparently, XPF-ERCC1 is able to bind and partially open the products of its incision reaction, thereby generating new substrates for subsequent incision reactions. We also observed that XPF-ERCC1 activity on the bottom strand is slightly lower (compare lanes 4-16 and 20-32), arguing for some sequence-specificity of XPF-ERCC1.

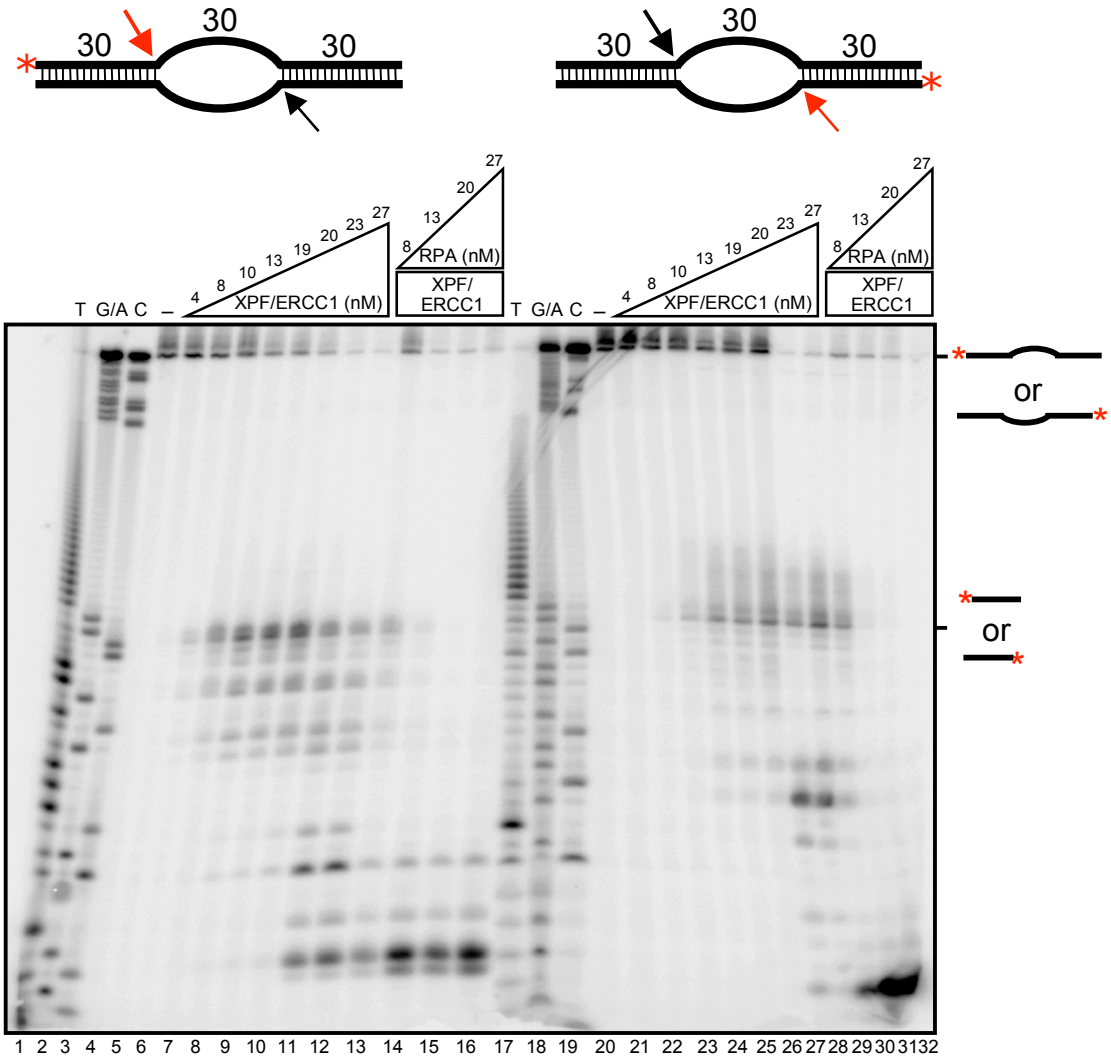

Fig.2. Effect of RPA on Endonuclease Activity of XPF-ERCC1 on a Bubble Substrate. 6.7 nM of a bubble substrate was incubated at 37°C for 45 minutes in presence of 2 mM $MgCl_2$ with wild-type XPF-ERCC1 and RPA. Lanes 4 and 20, no protein was added. Lanes 5-12 and 21-28, only XPF-ERCC1 was added. Lanes 13-16 and 29-32, XPF-ERCC1 and RPA were added. The concentrations of XPF-ERCC1 and RPA used are indicated on the top of the gel. The position of the 5' radiolabel on the substrate is indicated by a red asterisk, the positions of the XPF-ERCC1 incisions are marked with arrows, the red one representing the incision site on the labeled strand. The reaction products are shown on the right side of the gel. Sequencing ladders are shown for T (lanes 1 and 17), G/A (lanes 2 and 18) and C (lanes 3 and 19).

Presence of a Lesion on the Bubble Substrate and XPA do not Influence XPF-ERCC1 Activity

To test whether the presence of a damage on the bubble substrate might provide a binding site for XPA, which can then alter the XPF-ERCC1 activity or incision pattern in conjunction with RPA, we used a bubble with a dG-AF adduct at the position 50 of the bubble (Figure 3). Either the damaged (lanes 1-9) or undamaged (lanes 10-18) strand was 5' labeled. Overall XPF-ERCC1 activity was slightly lower on the bottom strand (compare lanes 2 and 6 with 11 and 15), as observed earlier without the damage (Figure 2), but the presence of XPA either without (compare the lanes 13 and 11 or 14 and 12, for example) or with the damage (compare the lanes 4 and 2 or 5 and 3, for example) did not influence the activity of XPF-ERCC1. To test whether XPG can influence XPF-ERCC1 incision activity, we performed the same experiment with the addition of XPG (data not shown), but we did not observe any change in the incision pattern or intensity. We conclude that in this experimental setup, presence of the DNA damage and/or XPA and XPG has no influence on the XPF-ERCC1 activity. Under these conditions, only RPA is able to change XPF-ERCC1 activity.

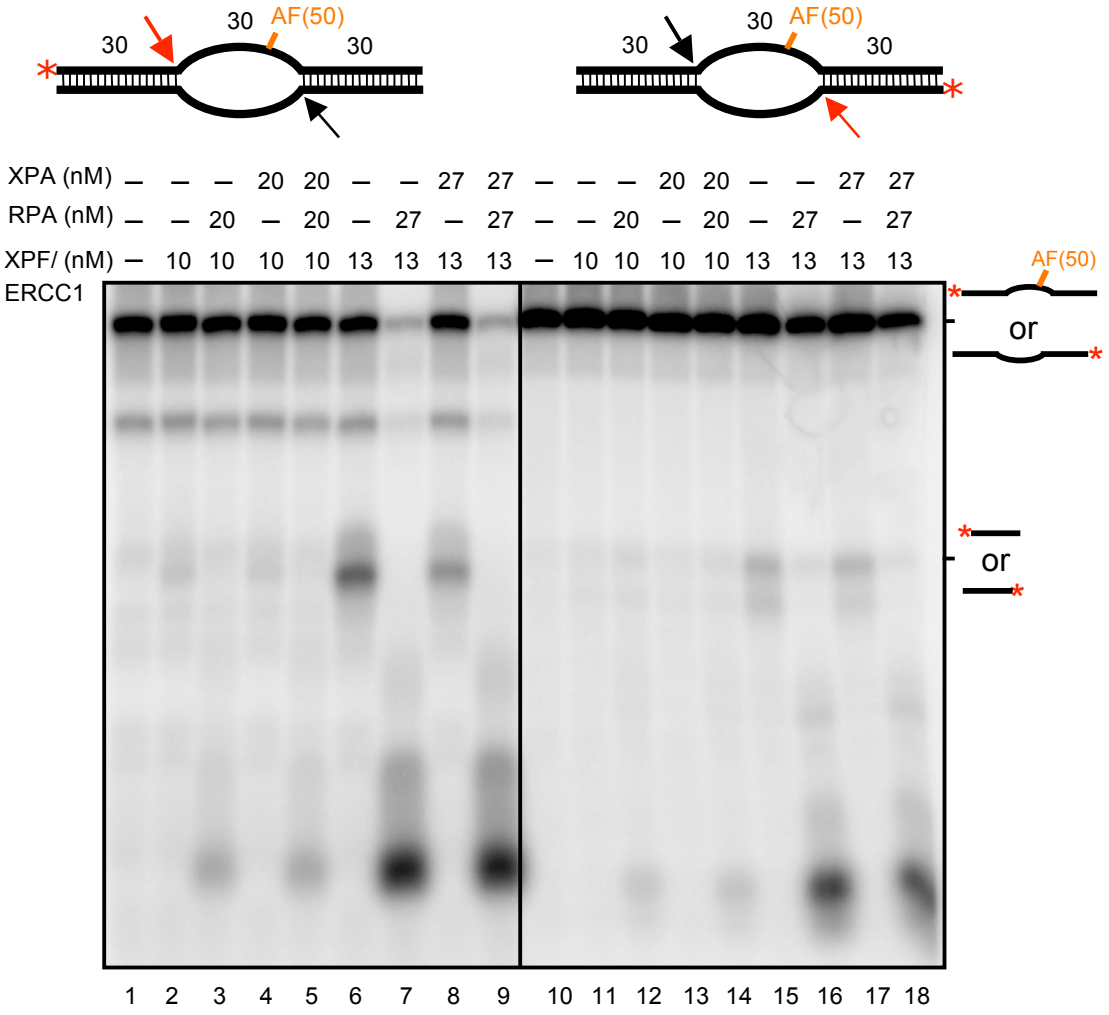

Fig.3. XPA does not Influence Endonuclease Activity of XPF-ERCC1 on a Bubble Substrate with a Damage. 6.7 nM of a bubble substrate was incubated at 37°C for 45 minutes in presence of 2 mM $MgCl_2$ with wild-type XPF-ERCC1, RPA and XPA. Lanes 1 and 10, no protein was added. Lanes 2, 6, 11 and 15, only XPF-ERCC1 was added. Lanes 3, 7, 12 and 16, XPF-ERCC1 and RPA were added. Lanes 4, 8, 13 and 17, XPF-ERCC1 and XPA were added. Lanes 5, 9, 14 and 18, XPF-ERCC1, RPA and XPA were added. The concentrations of XPF-ERCC1, RPA and XPA used are indicated on the top of the gel. The position of the 5' radiolabel on the substrate is indicated by a red asterisk, the positions of the XPF-ERCC1 incisions are marked with arrows, the red one representing the incision site on the labeled strand. The position of the dG-AF adduct is indicated. The reaction products are shown on the right side of the gel.

Substrate Dependent Stimulation of XPG by PCNA

It has been demonstrated previously that XPG and PCNA interact (Gary et al., 1997), but whether that interaction is needed for NER is still not clear. There are at least two possible events that could be influenced by the interaction

between XPG and PCNA: XPG could influence the recruitment of PCNA and/or PCNA could stimulate the 3' incision by XPG. Evans et al. have analyzed the effect of PCNA on XPG cleavage on a bubble substrate and found no effect (Evans et al., 1997).

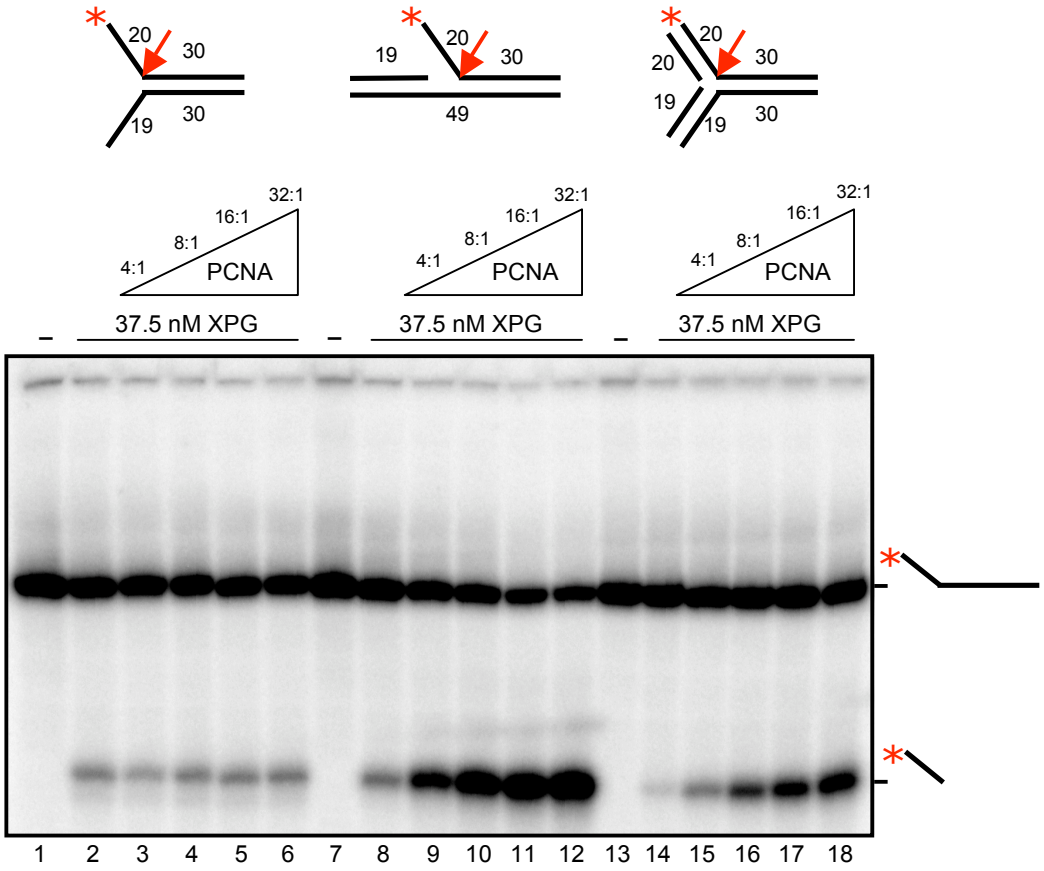

Fig.4. Stimulation of XPG cleavage by PCNA on Different Substrates. 2.5 nM of a DNA substrate was incubated at 30°C for 1 hour in presence of 2 mM $MgCl_2$ with wild-type XPG and PCNA. Lanes 1, 7 and 14, no protein was added. Lanes 2, 8 and 14, only XPG was added. Lanes 3-6, 9-12 and 15-18, XPG and PCNA were added. The concentrations of XPG used and the ratio of PCNA to XPG are indicated on the top of the gel. The position of the 5' radiolabel on the substrate is indicated by a red asterisk, the position of the XPG incision is marked with red arrows. The reaction products are shown on the right side of the gel.

We reasoned however (based on our results presented in the previous chapter) that a 5'-flap (Figure 4, the substrate in the middle) may be more likely what XPG encounters just before performing the 3' incision and therefore we examined the effect of PCNA on XPG incision using a 5' flap substrate and two similar substrates which were previously shown to be processed by XPG (Hohl et al., 2003). While there was no influence of PCNA on XPG incision on a splayed arm substrate (lanes 2-6), PCNA strongly stimulated XPG cleavage on the 5' flap substrate (lanes 8-12) and on the three-way junction with a nick (lanes 14-18).

XPG FF-AA Mutant Deficient in the Interaction with PCNA does not Stimulate XPG Incision

To test the specificity of the effect of PCNA, we generated an XPG protein with a mutated PIP box F996A/F997A (FF-AA). An analogous mutant of FEN-1 (F343A/F344A) did not bind PCNA and failed to stimulate excision in long-patch base excision repair (Gary et al., 1999). The stimulation observed with the wild type XPG (Figure 5, lanes 3-6) was almost completely abolished with the FF-AA mutant (lanes 9-12).

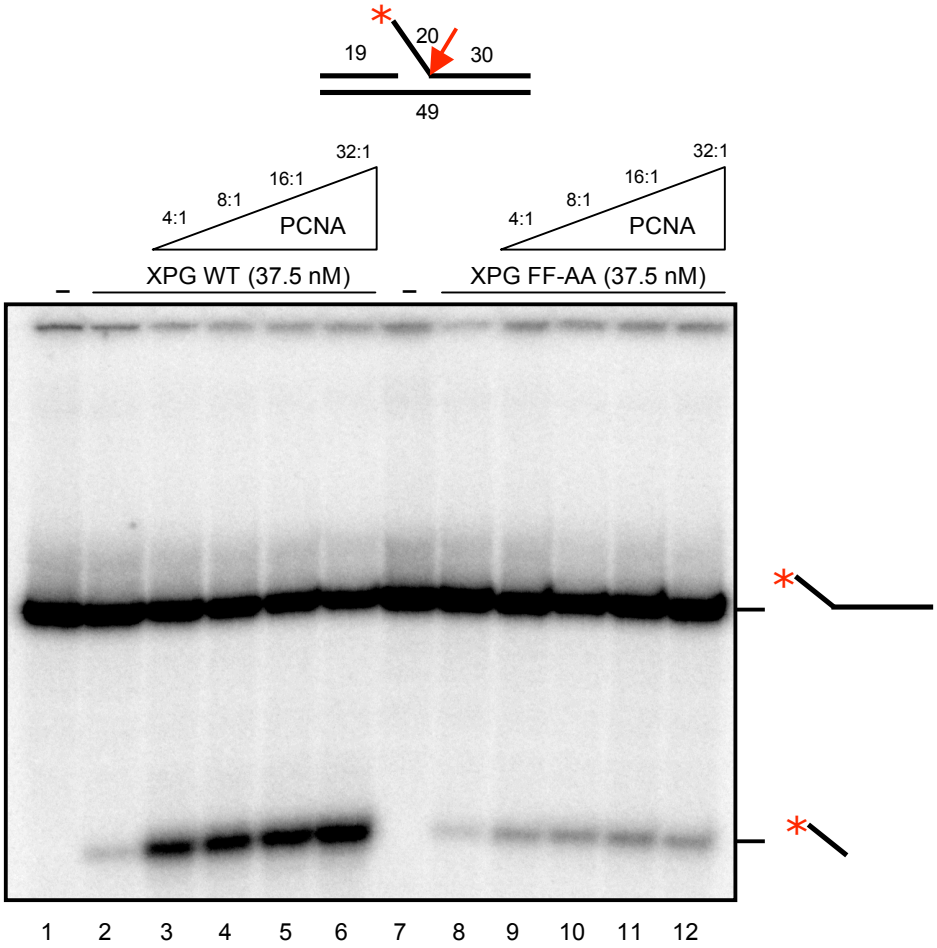

Fig.5. Stimulation of XPG cleavage by PCNA is Abolished in the XPG FF-AA Mutant deficient for PCNA Binding. 2.5 nM of a DNA substrate was incubated at 30°C for 1 hour in presence of 2 mM $MgCl_2$ with wild-type and FF-AA XPG and PCNA. Lanes 1 and 7, no protein was added. Lanes 2 and 8, only XPG was added. Lanes 3-6 and 9-12, XPG and PCNA were added. The concentration of XPG used and the ratio of PCNA to XPG are indicated on the top of the gel. The position of the 5' radiolabel on the substrate is indicated by a red asterisk, the position of the XPG incision is marked with a red arrow. The reaction products are shown on the right side of the gel.

XPG FF-AA is able to Complement XP-G Cell Extracts in in vitro NER

Excision and Repair Synthesis

As FEN-1 FF-AA mutant failed to stimulate excision in long-patch base excision repair, we wanted to test the influence of the FF-AA mutation in XPG on NER *in vitro.* Closed circular DNA with a site-specific cisplatin lesion was

incubated with a XP-G extract in repair buffer and the excision of the damaged oligonucleotide was detected by annealing a complementary DNA strand with a G_4 overhang and fill in with Sequenase 2.0 and [α-^{32}P]dCTP (Moggs et al., 1996). While no excision was observed in the XP-G extract, addition of wild-type XPG and XPG FF-AA led to the formation of a ladder of oligonucleotides specific for the NER *in vitro* reaction (Figure 6, lanes 3 and 4). Therefore, mutation of the PIP box in XPG did not affect the excision step in NER.

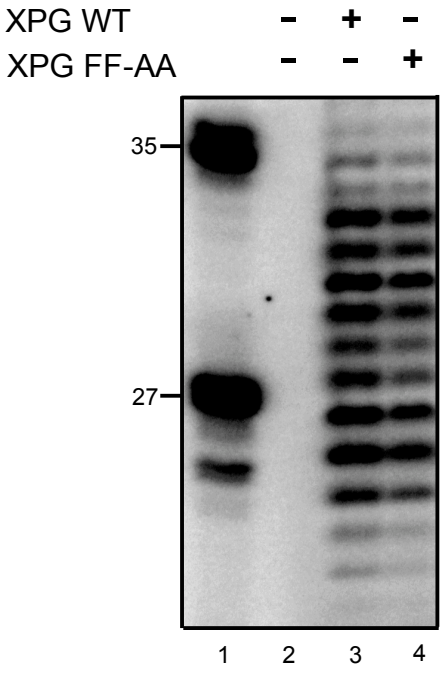

Fig.6. FF-AA Mutant XPG Protein Complements the Double Incision Defect of a XPG-deficient Cell Extract. Cell extracts prepared from the XPG-deficient fibroblasts XPCS1RO were incubated with a plasmid containing a single 1,3-cisplatin adduct in absence of protein (lane 2) or in presence of 730 fmol wild-type (lane 3) or FF-AA (lane 4) recombinant XPG protein. The excision products containing the cisplatin adduct were detected by annealing of an oligonucleotide complementary to the excised oligonucleotides with a 5' GGGG overhang serving as a template for end labeling with [α-^{32}P]dCTP and separated on a 14% denaturing polyacrylamide gel and visualized by autoradiography. A *Msp*I digest of pBR322 (lane 1) served as a size marker.

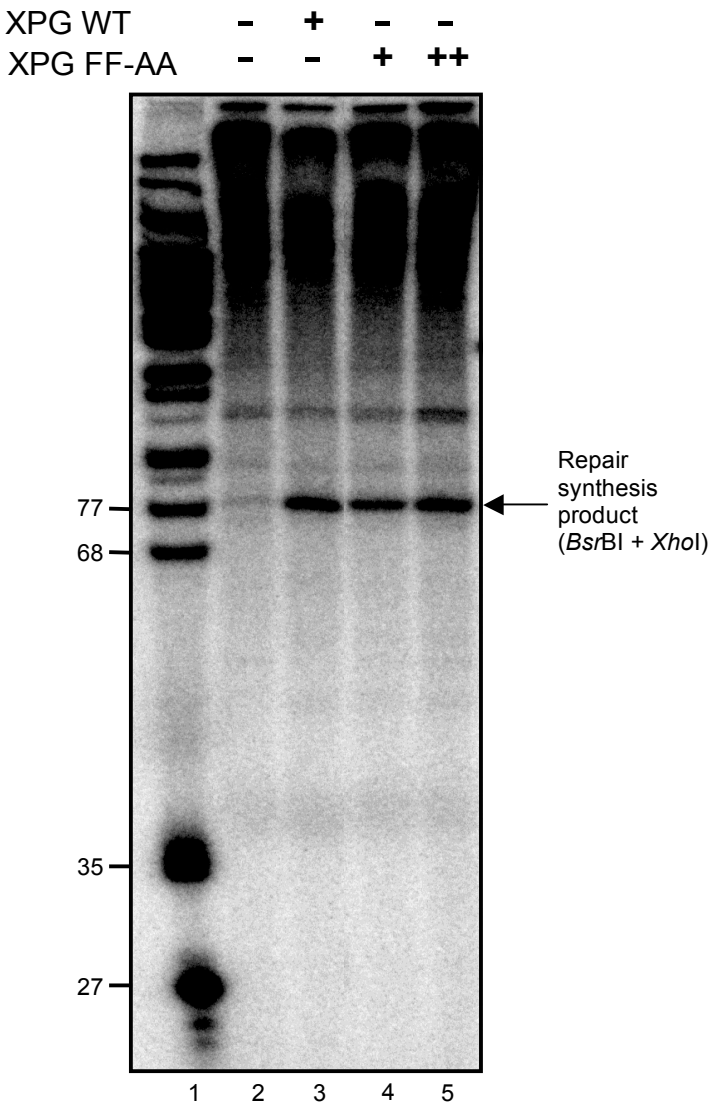

Fig.7. FF-AA Mutant XPG Protein Complements the Repair Synthesis Defect of a XPG-deficient Cell Extract. Cell extracts prepared from the XPG-deficient fibroblasts XPCS1RO were incubated with a plasmid containing a single 1,3-cisplatin adduct in absence of protein (lane 2) or in presence of 600 fmol wild-type (lane 3), 600 fmol FF-AA (lane 4) or 1.7 pmol FF-AA (lane 5) recombinant XPG protein as well as 10 µCi of [α-^{32}P]dCTP and 10µCi [α-^{32}P]TTP. DNA was further purified, digested with *Bsr*BI+*Xho*I and analyzed on a denaturing PAGE gel. A *Msp*I digest of pBR322 (lane 1) served as a size marker.

Next we have looked at repair synthesis *in vitro* to examine the influence of the XPG-PCNA interaction on the PCNA-dependent DNA synthesis to replace the damaged oligonucleotide. The DNA with a defined cisplatin lesion was incubated with a XP-G extract in repair buffer in the presence of [α-^{32}P]dCTP and [α-^{32}P]TTP with or without purified wild-type or PIP-mutant XPG. After the reaction, DNA was purified and cleaved with *Bsr*BI and *Xho*I to look specifically at incorporation of radioactive dCTP and TTP at the site of the damage resulting in specific labeling of the DNA fragment of 79 nucleotides. Incubation with XP-G extract alone resulted only in the appearance of a background signal (Figure 7, lane 2). With the addition of the wild-type XPG (lane 3) and XPG FF-AA (lanes 4 and 5), a strong band of similar intensity was observed arguing for no major effect of XPG-PCNA interaction on DNA repair synthesis step of NER. Thus, a mutation of the PIP box in XPG does not significantly affect the repair synthesis step of NER *in vitro*.

DISCUSSION

Need for a Precise Regulation of the Assembly of NER Complexes

Although controversially discussed in the past, it is now widely accepted that NER factors assemble sequentially at sites of DNA damage (Guzder et al., 1996; Hoogstraten et al., 2002; Houtsmuller et al., 1999; Mu et al., 1997; Rademakers et al., 2003; Riedl et al., 2003; Wakasugi and Sancar, 1998; Zotter et al., 2006). The sequential assembly implies the need for a precisely regulated

recruitment of different NER proteins to damaged sites through protein-protein interactions. We asked the question whether there are additional functions of the established protein-protein interactions among the NER factors in addition to recruitment of subsequent factors. In fact, several of such functions have already been established: XPF and ERCC1 stabilize each other when in complex (Biggerstaff et al., 1993; van Vuuren et al., 1993), HR23B stabilizes XPC (Ng et al., 2003) and XPG stabilizes TFIIH complex (Ito et al., 2007). We were particularly interested in exploring whether and how different NER factors modulate the activities of the two endonucleases, XPF-ERCC1 and XPG.

Influence of Early NER proteins on the 5' incision by XPF-ERCC1

The previous studies that analyzed the influence of RPA on the XPF-ERCC1 and XPG activity produced somewhat controversial results. Both stimulation and inhibition of the both endonucleases have been reported (Bessho et al., 1997; de Laat et al., 1998; Evans et al., 1997; Matsunaga et al., 1996). Although one study showed how RPA can change its inhibitory/stimulatory effect depending on how it is positioned on the ssDNA relative to the ssDNA/dsDNA junction (de Laat et al., 1998), it still does not explain the discrepancies in the results of the experiments in which the same substrate (bubble DNA) was used. Our main interest was to see what happens if we use a bubble with a damage, mimicking a NER reaction intermediate, as we hypothesized that in this case XPA bound to the damage might stimulate XPF-ERCC1 incision or change the incision sites, possibly leading to less incised sites. However, we observed that neither the presence of the lesion, XPA or the lesion and XPA together had any

influence on the XPF-ERCC1 cleavage, which was stimulated only by RPA (Figures 1, 2 and 3). In light of more recent findings that XPA might not recognize the DNA damage itself, but possibly altered DNA conformations (Camenisch et al., 2006), the most obvious interpretation is that the model substrate which we used for our studies is likely not what XPA encounters *in vivo*.

Influence of Late NER proteins on the 3' incision by XPG

XPG is one of the latest proteins to dissociate from the damaged DNA after the dual incision, possibly only after the arrival of the repair synthesis factors (Riedl et al., 2003). Therefore it might be involved in the tight coupling of the two steps, possibly through its interaction with PCNA (Gary et al., 1997). However, the importance of that interaction for NER is still not clear as the mutation in XPG, which almost completely abolishes PCNA binding, leads only to mild UV-sensitivity (Gary et al., 1997). Additionally, an attempt to observe a PCNA-dependent stimulation of XPG cleavage, as it was observed for FEN-1 (Wu et al., 1996), has failed (Evans et al., 1997). On the contrary, here we have observed a strong stimulation of XPG activity by PCNA on a 5' flap substrate, which might share features with an intermediate that XPG cleavage encounters in the context of NER *in vivo* (see Chapter 2). The stimulation was abolished when a mutant XPG protein, deficient in its interaction with PCNA, was used. However, that mutant did not have significantly impaired activity in *in vitro* NER excision and repair synthesis. The question remains whether the interaction between XPG and PCNA is important for NER at all. It is still possible that, as it is the case with FEN-1 (Sakurai et al., 2005), XPG interacts with PCNA through

more than one domain and only the disruption of all the interaction motifs would have an observable effect.

EXPERIMENTAL PROCEDURES

Protein Purification

XPF wild-type, XPG wild-type and XPG FF-AA proteins were expressed in Sf9 insect cells and purified as described previously (Enzlin and Schärer, 2002; Hohl et al., 2003). Typically 0.2-0.5 mg of proteins were obtained at concentrations of 0.2-0.3 mg/ml. RPA protein was expressed and purified as previously described (Henricksen et al., 1994). The final concentration was 0.07 mg/ml. XPA protein was expressed in E.coli BL21(DE3)/LysS from pET15b-XPA and purified by Ni^{2+}-NTA, gel filtration, and heparin chromatography to the final concentration of 0.17 mg/ml. PCNA (3.5 mg/ml) was a gift from Pavel Janscak, IMCR, University of Zurich, Switzerland.

Oligonucleotides

Oligonucleotides used were either synthesized on an Expedite 8909 nucleic acid synthesis system or purchased from Metabion (Germany) and purified by denaturing PAGE. 5' end labeling was carried out using T4 polynucleotide kinase and [γ-^{32}P]ATP. For 3' labeling, the oligonucleotides were incubated with terminal deoxynucleotidyl transferase and [α-^{32}P]ddATP. The labeled oligonucleotide was annealed with the complementary strand in annealing buffer (50 mM NaCl, 10 mM Tris-Cl, pH 8.0) by heating for 5 min at 95

°C and cooling to room temperature. Substrates were annealed with a 3-fold excess of the unlabeled complementary strand. The sequences of the oligonucleotides used were as follows: A20, 5'-TCAAAGTCACGACCTAGACACTGCGAGCTCGAATTCACTGGAGTGACCTC; B19, 5'-GAGGTCACTCCAGTGAATTCGAGCTCGCAGCAATGAGCACATACCTAGT; C19, 5'-ACTAGGTATGTGCTCATTG; D20, 5'-TGTCTAGGTCGTGACTTTGA. Oligonucleotides A20 and B19 were annealed to form a splayed-arm substrate; to form a 5'-flap substrate, oligonucleotides A20, B19 and C19 were annealed and to form a three-way junction substrate with the nick at the junction, oligonucleotides A20, B19, C19 and D20 were annealed. For the 90-mer bubble substrate with a bubble size of 30 nt, strand 1 (5'-CCAGTGATCACATACGCTTTGCTATTCCGGT$_{30}$CCGTGCCACGTTGTATGCCCACGTTGACCG) was annealed with the strand 2 (5'-CGGTCAACGTGGGCATACAACGTGGCACGGT$_{30}$CCGGAATAGCAAAGCGTATGTGATCACTGG).

Nuclease Assay

For XPG, 50 fmol of 5' or 3' ^{32}P end-labeled DNA substrate was incubated with XPG and PCNA in nuclease buffer (25 mM Tris-Cl, pH 6.8, 10% glycerol, 2.5 mM β-mercaptoethanol, 0.5 mg/ml bovine serum albumin, 30 mM KCl) in the presence of 2 mM $MgCl_2$ in a reaction volume of 20 µl for 1 hour at 30°C. The reactions were stopped by the addition of an equal volume of formamide loading buffer (90% (w/v) formamide, 0.5x TBE, 0.1% bromphenol blue, and xylene cyanol FF) and heating for 5 min at 95 °C. The samples were loaded onto a 12%

(cross-linking ratio 19:1) denaturing polyacrylamide gel containing 1x TBE and 7 M urea and run for 1 hour at 600 V. The bands were visualized by autoradiography, using Typhoon 9400 PhosphorImager (Amersham Biosciences).

For XPF-ERCC1, nuclease reactions (15 μl) were performed in 25 mM HEPES pH 8.0, 40 mM NaCl, 10% glycerol, 0.5 mM β-mercaptoethanol, 0.1 mg/ml bovine serum albumin (BSA) and 0.4 mM $MnCl_2$. Reaction mixtures contained 100 fmol of DNA substrate and different amounts of XPF-ERCC1, RPA and XPA proteins, and were incubated at 37°C for 45 minutes. Reactions were stopped by adding 15 μl of loading dye (90% formamide/10 mM EDTA) and heating at 95°C for 5 min. Samples were loaded onto 10 or 12% (19:1) denaturing polyacrylamide gels containing 0.5 times TBE and run for 1 h at 550 V or 1.5 hours at 55W for the sequencing gel. Reaction products were visualized by autoradiography, using Typhoon 9400 PhosphorImager (Amersham Biosciences).

In Vitro NER Dual Incision Assay

Covalently closed circular DNA (pBluescript SK II (+)) containing a single 1,3-intrastrand d(GpTpG) cisplatin-DNA crosslink was prepared as described (Moggs et al., 1996) and additionally purified over two consecutive sucrose gradients. Reactions were carried out in a buffer containing 40 mM HEPES-KOH (pH 7.8), 70 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP, 0.36 mg/ml BSA, 22 mM phosphocreatine (di-Tris salt) and 50 ng/μl creatine phosphokinase. Each reaction contained 50 ng DNA and 30 μg of cell-free extract prepared from XPG-

deficient fibroblast cells (XPCS1RO). Complementation was assayed upon addition of 730 fmol wild-type or FF-AA XPG protein. Reactions were incubated at 30°C for 45 minutes. 50 nM of an oligonucleotide complementary to the excision product with a G_4 5'-overhang (5'-GGGGGAAGAGTGCACAGAAGAAGACCTGGTCGACC) was added, followed by heat inactivation at 95°C for 5 minutes. After cooling down the reactions for 15 minutes at room temperature, 0.5 units of Sequenase and 3.5 μCi of [α-^{32}P]dCTP (both from Amersham-Pharmacia, diluted in Sequenase dilution buffer) were added. Reactions were incubated for 3 minutes at 37°C prior the addition of 1.2 μl dNTP mix (100 μM of each dATP, dGTP, TTP and 50 μM dCTP) and incubation for another 12 minutes at 37°C. Reactions were stopped by addition of formamide loading buffer, heated at 95°C for 5 minutes and analyzed on a denaturing polyacrylamide gel. The gel was exposed on a phosphor screen and scanned on the PhosphorImager.

In Vitro NER Repair Synthesis Assay

The assay was performed using the same substrate, cell extracts and proteins as described for the excision assay with minor modifications. The reactions contained 10 μM dATP, 10 μM dGTP, 5 μM dCTP, 5 μM TTP, 10 μCi of [α-^{32}P]dCTP and 10μCi [α-^{32}P]TTP. Complementation was assayed upon addition of 600 fmol of wild-type or mutant protein (XPG wild-type or FF-AA). Reactions were incubated at 30°C for 3 hours. DNA was purified using MinElute PCR Purification Kit (Qiagene) and cleaved with *Bsr*BI and *Xho*I.

Cell Culture Conditions and Preparation of Whole Cell Extracts

For the generation of whole cell extracts, SV40-transformed fibroblast cells XPCS1RO (XPG deficient, (Ellison et al., 1998)) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal calf serum and 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in the presence of 5% CO2. Cells were grown to near confluency, and whole cell extract was prepared accordingly to a published procedure (Biggerstaff and Wood, 1999).

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Conclusions and Future Directions

The critical catalytic events in nucleotide excision repair are the two incisions 5' and 3' to the lesion by the XPF-ERCC1 and XPG proteins, respectively, leading to the removal of an oligonucleotide containing the lesion and transition to the repair synthesis step to restore the original DNA sequence. Uncoupling of the two steps, double incision and repair synthesis, would result in formation of a highly recombinogenic ssDNA gap, suggesting that the transition between the two steps has to be precisely regulated. So far the only experimental evidence for a possible mechanism coordinating the two steps was the observation of similar kinetics of the excision of the damaged oligonucleotide and the repair synthesis. The only clue about the nature of such a mechanism were the experiments demonstrating a sequential assembly and, equally important, disassembly of the NER complexes at the site of the lesion, with some early NER proteins (RPA and XPG) staying longer at the damaged sites and possibly being involved in the recruitment of DNA replication machinery through protein-protein interactions.

Work in this thesis has demonstrated a close connection between the two steps at the mechanistic level, showing that double incision is not required for the initiation of repair synthesis, which can start already after the cleavage 5' to the lesion by XPF-ERCC1. We therefore suggest that a temporal order of incisions with the 5' incision preceding the 3' incision ensures the excision of the damaged

oligonucleotide only when the repair machinery has already started to fill in the DNA gap.

Although we have clearly shown that repair synthesis can start without XPG cleavage, it still has to be seen whether that actually happens in the wild-type situation. Direct evidence for our model would be the existence of repair intermediates containing both the damage and newly incorporated nucleotides as a result of partial repair synthesis. To isolate such intermediates, we plan to use fluorescently labeled NER substrates (developed in our laboratory by Jérôme Gualbert) in repair synthesis assay with radioactively labeled dNTPs. In that way we can at the same time detect the damage and the repair synthesis product. However, this method does not ensure that they are present in the same molecule so we will additionally label NER lesions with biotin to isolate such molecules, which have the damage, and the radioactive nucleotides.

Another way to look at NER intermediates without the need for mutant proteins is through modifications of phosphodiester bonds. It is possible to replace individual oxygen atoms of the phosphodiester bond, which will be cleaved by one of the two endonucleases by sulfur. Mg^{2+}, which is a cofactor for both endonucleases, does not bind sulfur ligands resulting in the blockage of the endonuclease, which encounters such a modified phosphodiester bond. Addition of Mn^{2+} allows the blocked endonuclease to cleave as it can bind to both oxygen and sulfur. That system, developed by Rolf Buff, Marcel Hohl and Jung-Eun Yeo, is expected also to contribute to understanding of the assembly and disassembly of NER machinery.

On longer term, one of the main future directions of the whole NER field will certainly be the investigation of posttranslational modifications of NER factors and the function of those modifications in NER pathway and possibly connection with other DNA repair pathways and/or signaling, DNA replication and transcription.

Abbreviations

NER	nucleotide excision repair
BER	base excision repair
APE1	apurinic/apyrimidinic endonuclease 1
XRCC3	X-ray repair cross-complementing group 3
CPD	cyclobutane pyrimidine dimer
6-4PP	6-4 photoproduct
XPC	xeroderma pigmentosum complementing group C
HR23B	homolog of RAD23 B
TFIIH	transcription factor II H
RPA	DNA replication protein A
XPA	xeroderma pigmentosum complementing group A
XPF	xeroderma pigmentosum complementing group F
ERCC1	excision repair cross-complementing group 1
XPG	xeroderma pigmentosum complementing group G
PCNA	proliferating cell nuclear antigen
RFC	replication factor C
GG-NER	global genome nucleotide excision repair
TC-NER	transcription-coupled nucleotide excision repair
CSA	Cockayne syndrome protein A
CSB	Cockayne syndrome protein B
MMR	mismatch repair

DSB	double-strand DNA break
HR	homologous recombination
NHEJ	non-homologous end joining
XP	xeroderma pigmentosum
CS	Cockayne syndrome
TTD	trichothiodystrophy
XPB	xeroderma pigmentosum complementing group B
XPD	xeroderma pigmentosum complementing group D
UDS	unscheduled DNA synthesis
AF	aminofluorene
XPE	xeroderma pigmentosum complementing group E
DDB	damaged DNA binding protein
CAK	cyclin-activating kinase
CTD	carboxy-terminal domain
dsDNA	double-stranded DNA
ssDNA	single-stranded DNA
FEN-1	flap endonuclease 1
BD	binding domain
CAF-1	chromatin assembly factor 1
XPV	variant form of xeroderma pigmentosum
PIP	PCNA-interacting peptide