Nucleotide excision repair : complexes and complexities : a study of global genome repair in human cells

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All we have to decide is what to do with the time that is given us

J.R.R. Tolkien - the Lord of the Rings

aan mijn vader

voor mijn moeder

Contents

Aim and outline of the thesis

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Of all exogenous agents that damage genomic DNA and hence present a threat to the integrity of its genetic information, the ultraviolet B (UVB) component of sunlight possesses high clinical relevance because of its abundance. UVB induces predominantly two types of photolesions: cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4 photoproducts or 6-4PP). Uniquely, placental mammals rely solely on the nucleotide excision repair (NER) system to repair these photolesions. In addition, NER is capable of removing a wide range of bulky, helix-distorting lesions, including numerous chemical adducts such as those caused by the anticancer drug *cis*-diamminedichloroplatinum(II) (cisplatin), the poison gas nitrogen mustard and polycyclic aromatic hydrocarbons found in burnt food and cigarette smoke. The relevance of the NER pathway, particularly in the removal of UV-induced photolesions, is emphasised by the existence of three inherited disorders caused by mutations in genes encoding NER proteins. Patients suffering from these disorders invariably display hypersensitivity to sunlight, and patients suffering from one disease, xeroderma pigmentosum, combine this with a dramatically increased risk of developing skin cancers.

In the first part of this thesis, the different cellular defence mechanisms against various types of DNA damage are described. Chapter 1 summarises the possible reactions of a cell to DNA damage and briefly sketches the DNA repair pathways. Chapters 2 and 3 concern themselves solely with NER: chapter 2 introduces some background important to understanding NER while chapter 3 provides a detailed step-by-step description of the global genome repair pathway, highlighting the roles of every factor involved. Subsequently chapter 4 elaborates on the inhibition of transcription that is observed after the introduction of NER-types of DNA damage. Chapter 5 puts NER in a nuclear perspective, describing its interplay with chromatin, the natural substrate of any DNA repair pathway.

The research presented in this thesis is focused on various aspects of the NER pathway in live cells. One of the main questions addressed is whether the NER complex assembles on the lesion in a sequential manner or whether it pre-exists as a so-called 'repairosome'. Experimental evidence as described in chapter 6 suggests that a sequential assembly of NER proteins on DNA lesions is most likely. The technique of local UV irradiation used in this study was utilised to address another question: the possible involvement of transcription factor TFIIH in the UV-induced temporary inhibition of transcription. The result of this research, together with a detailed description of the local irradiation method, is presented in chapter 7. The cellular characteristics of XPA, an NER protein involved in damage verification were further investigated in chapter 8. In contrast to what had been reported previously, we found that XPA does not form a cellular complex with the single-strand binding protein RPA. After having investigated the functioning of essential NER proteins, the role of the accessory damage recognition factor UV-DDB in NER was assessed. Using a significantly lower UV dose compared to that used in earlier published research, we discovered a previously ill-recognised role for UV-DDB in 6-4PP repair which is described in chapter 9. Turning the attention to the later stages of the NER reaction, the involvement of DNA polymerases δ and ε and DNA ligase I in the DNA resynthesis step of NER *in vivo* was investigated, while concurrently the stability of the preincision NER complex and the DNA resynthesis complex were assessed. The results of this research are presented in chapter 10.

Chapter 1

Cellular responses to DNA damage

1 Cellular responses to DNA damage

Despite the fact that the information stored in the genome is very stable from generation to generation, the physical carrier of this information, the DNA itself, is not completely inert at the same timescale. Rather, it continually deteriorates spontaneously and is exposed to numerous endogenous and exogenous agents that damage it. These alterations to the DNA directly threaten cellular survival by interfering with processes such as replication and transcription. Indirectly the cell is endangered when damages lead to mutations or chromosomal aberrations, which in turn can lead to cellular malfunctioning or worse, cell death. In higher multicellular organisms like humans, the formation of cancer is a particular outcome of cellular malfunctioning that threatens survival not of the cell, but of the entire organism. Cells respond to the infliction of damage to their DNA by activating a DNA damage signalling cascade and inducing various DNA repair mechanisms.

1.1 Cell cycle arrest

When DNA damage is present at various checkpoints during the cell cycle (the G1/S checkpoint at the entry of S-phase and the G2/M checkpoint at the transition from G2- to M-phase) this will result in a cell cycle arrest. The G1/S-phase arrest is especially important as this arrest prevents the cell from attempting to replicate damaged DNA. Such an attempt would not only risk the introduction of mutations but moreover the stalling of the replicative machinery on DNA lesions or even the abortion of replication in an attempt to replicate across a double-strand DNA break. The latter event is especially deleterious and is an important source of genomic instability, giving rise to chromosomal aberrations such as loss of heterozygosity. Arresting its cell cycle not only prevents the cell from this major harm, it also gives the opportunity for 'damage assessment'. If the DNA is deemed to be damaged beyond repair, a cell may commit to apoptosis, the process of regulated cell death. On the other hand, the cell possesses a variety of DNA repair systems that are capable of repairing many different types of DNA damages. If and when repair has been completed, the cell may resume its cell cycle. The following chapter briefly describes the various mechanisms that exist to repair damaged DNA.

1.2 DNA repair mechanisms

Direct reversal

The easiest way to deal with DNA lesions is to convert the modified nucleotides back to their original structure without even removing them from the sugar-phosphate backbone. Two major repair systems use this so-called direct reversal mechanism.

First, photoreactivation mediated by photolyases restores the UV-induced CPD or 6-4PP to their original pyrimidines using energy from absorbed light. The photolyase uses a first chromophore as light-harvesting antenna to capture a photon of appropriate wavelength. The resulting excitation energy is transmitted to a second chromophore positioned near the photolesion, and the photolesion is subsequently split into its original two nucleotides. Typically, photolyases are specific for a single type of photolesion, targeting either CPD or 6-4PP but not both. Photolyases

are found in a range of species from bacteria and lower eukaryotes to plants and many animals including marsupials, but placental mammals are curiously devoid of this system.

The second example of direct reversal is the enzyme methylguanine DNA methyltransferase (MGMT) which is present in virtually all species throughout all kingdoms. It restores O^6-methylguanine back to guanine by transferring the methyl group onto a cysteine in its active site. The formed covalent methyl-cysteine bond is stable, inactivating the enzyme after only one round of repair; hence, MGMT is referred to as a suicide enzyme.

Base excision repair and single-strand break repair

Base excision repair (BER) deals with relatively mild DNA helix-distorting lesions that for a large part are produced by the cell's mitochondrial respiration processes, resulting in lesions such as 7,8-dihydro-8-oxoguanine (8-oxoguanine) and 5,6-dihydro-5,6-dihydroxythymine (thymine glycol). Other endogenous lesions tackled by BER include methylated bases, apurinic/apyrimidinic (AP)-sites formed after the spontaneous cleavage of a sugar-base bond, and single-strand breaks formed after a cleavage of the sugar-phosphate chain. Finally also uracil, which is introduced in DNA by the spontaneous deamination of methylated cytosine, is targeted by BER. These lesions are formed at high rates; estimates of the number of lesions that BER processes daily in a human cell range from ten thousand for oxidative damages only (Ames et al., 1993) to one million in total (Holmquist, 1998). To recognise the various base damages, BER employs an array of damage-specific glycosylases, including enzymes that recognise oxidised pyrimidines (8-oxoguanine glycosylase, thymine glycol glycosylase) and alkylated purines (methyl-purine glycosylase). Uracil is recognised specifically by uracil-DNA glycosylase.

Depending on the nature of the lesion and the glycosylase involved, BER can proceed via a number of routes. Firstly, the lesion may be processed by a glycosylase with 3'-β-lyase activity (a bifunctional glycosylase). This type of glycosylase cleaves off the damaged base and subsequently also incises the sugar-phosphate backbone 3' to the resulting AP site. 3' trimming by pol β or the HAP1 enzyme removes the remaining sugar residue; pol β then inserts the correct nucleotide followed by recruitment of XRCC1 and DNA ligase III, the latter of which ligates the nick. As the repair patch is only 1 nt long this pathway is referred to as short-patch BER. Secondly, when the glycosylase has no associated 3'-β-lyase activity (a monofunctional glycosylase), after the creation of the AP-site, the sugar-phosphate backbone is cleaved by HAP1 or pol β on the 5' side of the sugar. From here, BER can proceed via a short-patch or a long-patch repair pathway, depending on the nature of the damage. An unmodified AP-site will result in short-patch repair: pol β inserts 1 nt while displacing the sugar, followed by recruitment of XRCC1 and DNA ligase III and ligation of the nick. A reduced or oxidised AP-site however will lead to long-patch repair, a process involving pol β, pol δ or pol ε in combination with the replication factors RF-C and PCNA (and possibly RPA), where a patch of 2-10 nt is displaced while new nt are inserted. The resulting single-strand 'flap' is cleaved off by FEN1, after which the nick is sealed by DNA ligase I. For an extensive review of BER, the reader is referred to Barnes and Lindahl (2004).

A similar mechanism is employed in repair of single-strand breaks. These are recognised, bound and protected from recombination by the poly(ADP-ribose)polymerase 1 (PARP1) protein. After PARP1 has bound possible 'dirty' ends are trimmed off and XRCC1 is recruited. This protein then recruits proteins such as DNA polymerase β and DNA ligase III, and repair is completed via the latter stages of the BER pathway.

Double-strand break repair

Among the most cytotoxic DNA lesions are double-strand breaks (DSB), which can be formed either exogenously by high-energy radiation, or endogenously during mitotic recombination or during V(D)J recombination. As little as one DSB can cause cell death during mitosis; it is therefore of vital importance to the cell to repair any DSB that might arise. To quickly eliminate these lesions different DSB repair (DSBR) mechanisms are operational; i.e. non-homologous end joining (NHEJ) and homologous recombination (HR).

In the process of NHEJ, the ends of the broken strands are bound by the Ku70/Ku80 heterodimer, which attracts DNA-PKcs. In addition, a role for the Rad50-Mre11-Nbs1 protein complex in NHEJ was suggested but is as yet controversial, i.e. to bring the damaged ends together (de Jager et al., 2001). A defect in the Artemis protein gives rise to defective NHEJ and genomic instability (Moshous et al., 2001; Rooney et al., 2003) and Artemis is therefore thought to be involved in trimming of termini that require processing, i.e. break ends containing base damages (or hairpins in V(D)J recombination) (Jeggo and O'Neill, 2002; Riballo et al., 2004), before the break ends are rejoined by DNA-PKcs. Finally, the ends are ligated by the DNA ligase IV-XRCC4 heterodimer. Any processing of breaks prior to ligation occurs at the expense of losing genetic information (a few nucleotides); consequently the process of NHEJ can be considered as an error prone DSB repair process.

HR is a complex reaction that uses the intact information on the sister chromatid or the sister chromosome to restore information lost in the break. Firstly, the ends of the break are bound by Rad52 protein, possibly following end trimming by the Rad50-Mre11-Nbs1 (RMN) complex. This process gives rise to single-strand regions with 3' overhangs, on which Rad51 forms nucleoprotein filaments that search for homologous duplex DNA. Here, the RMN complex may also function in bringing an end together with an intact sister chromatid (de Jager et al., 2001). Once regions of homology have been found, the DNA strands are exchanged in a process that requires the Rad52, Rad54 and Rad55/57 proteins as well as the single-strand binding protein RPA. A DNA synthesis process followed by ligation restores the missing information and finally the crossed DNA strands are resolved to yield two DNA duplexes.

Finally, single-strand annealing (SSA) is a process that trims off the ends of a DSB with an exonuclease – possibly the RMN complex – until homologous regions are exposed on both sides of the break. These are then paired and the ssDNA overhangs cleaved off so that the ends can be ligated. RPA and Rad52 are likely candidates to participate in SSA. SSA can be considered as a subpathway of HR.

Unicellular organisms and mammalian stem and germ cells require their genetic information to remain intact, and therefore tend to rely more on the error-free HR pathway to repair DSB. On the other hand the less complex but error-prone NHEJ can be utilised without great risk by differentiated somatic cells. Even so, the use of HR is usually restricted to S-phase, when the sister chromatid is nearby to be used as a template. Outside S-phase, misalignment of repetitive DNA sequences might lead to deletions or translocations and hence the use of NHEJ tends to prevail.

Nucleotide excision repair

Nucleotide excision repair (NER) is the most versatile repair pathway because of its broad substrate specificity. NER is responsible for the removal of bulky, helix-distorting lesions from DNA. These lesions can result from a wide variety of agents, including the UV component of sunlight (introducing CPD and 6-4PP), benzo[a]pyrene and N-acetoxy-2-acetylaminofluorene (NA-AAF) (giving rise to

bulky DNA adducts), and cisplatin (leading to intrastrand crosslinks). The basic mechanism of NER is evolutionarily highly conserved and is found in many species, from bacteria to mammals with even significant structural homology between yeast and mammalian NER factors.

The first stage of NER, up to and including dual incision, requires six factors and several consecutive steps can be discerned. First, the damage is recognised by the XPC-hHR23B heterodimer. The helix around the lesion is then opened by transcription initiation factor IIH (TFIIH) and possibly RPA, after which the damage is verified (or demarcated) by XPA. This is followed by dual incision by two structure-specific endonucleases, XPG and the ERCC1-XPF heterodimer, which incise the damaged strand on each side of the lesion. The resulting damage-containing oligomer (in humans of a typical length of 24-30 nucleotides) is then released. In the second (postincision) stage of NER resynthesis across the resulting gap occurs which requires several proteins: RPA; PCNA, the processivity factor for replicative DNA polymerases; RF-C to load PCNA onto the DNA; and either DNA pol δ or pol ε. Finally, DNA ligase I ligates the nick. Since the research in this thesis focuses on NER, the subsequent chapters describe NER in far greater detail.

Replication-associated responses

Postreplication repair

Despite the presence of various repair mechanisms, DNA lesions occasionally escape repair and persist into S-phase. Also when a cell is subject to DNA damage during S-phase, its DNA replication machinery will encounter DNA lesions, many types of which form a potent block for the replicative polymerases α, δ and ε. These polymerases possess high fidelity and processivity to faithfully duplicate the genome during S-phase, but are incapable of coping with damaged nucleotides, causing the replication fork to stall. This can have disastrous consequences, such as the introduction of double-strand breaks (see above). Moreover, replication blocks form a potent signal to induce apoptosis. To avoid the deleterious effects of blocked replication, cells utilise several damage tolerance pathways, which allow the disturbed replicative polymerases to bypass blocking lesions. The two main routes of this so-called postreplication repair (PRR) pathway are translesion synthesis (TLS) and damage avoidance. For recent reviews on this subject, see Friedberg (2005) and Prakash et al. (2005).

Translesion synthesis

During translesion synthesis, the replicative polymerase is temporarily replaced with a polymerase capable of synthesising across the lesion and extending from the resulting mispaired bases. The last few years a range of DNA polymerases involved in TLS has been discovered; among them DNA polymerases η, ι, κ and ζ, and Rev1, each displaying different TLS capabilities and fidelities (Prakash et al., 2005). Generally, TLS polymerases possess a lower fidelity on undamaged DNA than the dedicated replication polymerases, most likely as a direct consequence of their wider active site pocket, needed to accommodate non-Watson-Crick base pairs during the replication past DNA lesions. As a consequence, TLS polymerases have a higher chance of inserting the wrong nucleotide opposite an undamaged template. Similarly many TLS polymerases do not incorporate the correct nucleotide opposite a damaged template; thus TLS is generally considered an error-prone process, leading to an increase in mutation rate. One notable exception to this latter 'rule' is the insertion of the correct nucleotides, i.e. two adenines, opposite a thymine dimer by DNA pol η.

Damage avoidance: template switching and daughter-strand gap repair

In contrast to TLS, which is usually error-prone, two other damage tolerance systems exist that are essentially error-free. During template switching, the DNA polymerase temporarily uses the newly synthesised strand of the sister chromatid as a template. When it has reached a point beyond the lesion site on the original template, it switches back to resume normal replication. In the process of daughter-strand gap repair, the DNA polymerase dissociates from the template in the vicinity of the lesion, after which the gap is repaired via a homologous recombination mechanism. After the damaged DNA has thus been bypassed, normal replication resumes beyond the damage. Both these processes are still poorly characterised and little is known about the proteins involved; apart from the Rad5 protein, only PCNA and DNA polymerase delta are reported to be involved (Torres-Ramos et al., 2002).

It has recently become clear that in the decision which subpathway of PRR to use, covalent modifications of PCNA, i.e. ubiquitination and SUMOylation play an important role. Specifically, mono-ubiquitination on lysine 164 of PCNA, a modification that depends on the Rad6-Rad18 complex (Hoege et al., 2002), results in the switch to the error-prone TLS pathway via the recruitment of pol η (Kannouche et al., 2004; Watanabe et al., 2004). In a Rad5- and Mms2-Ubc13-dependent reaction, a monoubiquitin moiety can subsequently be extended to a polyubiquitin chain, resulting in the selection of error-free PRR pathways (Hoege et al., 2002).

Mismatch repair

The replicative DNA polymerases (α, δ and ε) have relatively high fidelity, resulting in only 1 error per 10^5-10^6 nucleotides copied (Roberts and Kunkel, 1996). Still, thousands of mismatched base pairs will thus be introduced by copying the $6x10^9$ nt genome. Moreover, several TLS polymerases, which are much less accurate, may participate in replication past unrepaired lesions. If the mismatches are not removed, a next round of replication could fix them as mutations, resulting in possible impaired cellular functioning, cancerous cell formation or even cell death.

Mismatch repair (MMR) specifically deals with nucleotides misincorporated during replication, replacing the incorrect nucleotide in the newly synthesised strand with the correct one. MMR also removes insertion/deletion loops (IDL) resulting from polymerase slippage. Two heterodimers are responsible for recognition of the various MMR substrates: mismatched base pairs are recognised by the hMSH2-hMSH6 heterodimer, larger IDL are recognised by hMSH2-hMSH3, while both factors are capable of recognising single base insertions/deletions. After binding to their substrates, the hMSH2-containing heterodimers attract a heterodimer consisting of hMLH1 and PMS2; hMSH2-hMSH3 alternatively can attract an hMLH1-hMLH3 dimer. The hMLH1-containing heterodimers in turn couple the recognition of the mismatch or IDL with later steps, i.e.: 1) strand discrimination, still a poorly understood process in most organisms except *E. coli*; 2) excision, a step which (at least in *E. coli*) can remove hundreds of nucleotides up to the strand discrimination signal and which in mammals employs EXO1, PCNA and the proofreading exonucleases of DNA pol δ or pol ε; and 3) resynthesis by DNA pol δ or pol ε and associated factors. For a recent review on mismatch repair, the reader is referred to Kunkel and Erie (2005).

Chapter 2

Nucleotide excision repair: background and concepts

2 Nucleotide excision repair: background and concepts

2.1 Two modes of NER: GGR and TCR

In vivo NER consists of two subpathways. The most prominent pathway is the global genome NER (GGR) pathway which removes lesions from the entire genome. As highlighted by the existence of the inherited disorder xeroderma pigmentosum (discussed in chapter 2.2), this pathway plays an important role in preventing the accumulation of mutations in our genomic DNA that ultimately give rise to cancer. Our view of NER has come about by putting pieces from various sources together, resulting in the highly detailed understanding of NER we have today.

Starting with the observation that patients suffering from XP were defective in repair replication of DNA (Cleaver, 1968) progress has been made in leaps and bounds. After some initial confusion, eight complementation groups were established (discussed in chapter 2.2). Subsequently, the genes responsible were one by one cloned (Legerski and Peterson, 1992; Mudgett and MacInnes, 1990; Tanaka et al., 1990; Thompson et al., 1994; Weber et al., 1988; Weeda et al., 1990), and the proteins they encoded were characterised. Only the *XPE* and *XPV* genes remained elusive for a longer time (chapter 3.1; Masutani et al., 1999).

The next major step forward in the research of NER was the reconstitution of GGR *in vitro* using cell-free extracts (Masutani et al., 1993; Sugasawa et al., 1993; Wang et al., 1993; Wood et al., 1988) or purified proteins (Aboussekhra et al., 1995; Araujo et al., 2000; Bessho et al., 1997; Mu et al., 1995), combined with a variety of damaged DNA templates. These experiments have resulted in a detailed understanding of the role of each protein or protein complex in the reaction and have led to the insight that the NER reaction proceeds through various stages. However, several aspects of GGR have proven difficult to investigate *in vitro*: repair from chromatinised DNA (discussed in chapter 5) and the organisation of the NER complex, i.e. the interdependence of proteins recruited to the site of a lesion and their precise order of being incorporated into the NER complex (discussed in chapters 3, 6, 8 and 10).

Transcription-coupled repair: an outline

While GGR is dedicated to the removal of lesions from the entire genome, regardless of their location, on the other hand transcription-coupled NER (TCR) is a specialized pathway that removes lesions from the transcribed strand of active genes only (Mellon et al., 1987; Venema et al., 1992), and depends strictly on ongoing transcription. The relevance of TCR lies in the rapid removal of lesions that block transcribing RNA polymerase II (RNA pol II), ensuring that the vital process of transcription is obstructed for as short a period as possible.

To a large extent GGR and TCR use the same set of proteins. The major difference between them lies in the initial step, i.e. recognition of the DNA lesion. To this end GGR employs the specialised damage recognition proteins UV-DDB and XPC-hHR23B, whereas in TCR the stalling of RNA pol II on a lesion is thought to initiate repair. TCR additionally requires the involvement of the CSA and CSB proteins and XPA binding protein 2 (XAB2).

The CSB protein and its role in TCR are relatively well-studied compared to the other two TCR-specific proteins, but nonetheless the exact role of CSB in TCR remains unclear. The CSB protein is 168 kDa in size and contains seven conserved helicase domains (Troelstra et al., 1992). CSB is a member of the SWI2/SNF2 superfamily of ATPases and harbours a DNA-dependent

ATPase activity (Citterio et al., 1998; Guzder et al., 1996a) which drives a chromatin remodelling activity (Citterio et al., 2000; see also chapter 5.2). Inactivation of this ATPase activity does not fully abolish complementation by CSB of the recovery of RNA synthesis (RRS; explained in chapters 2.2 and 4.1) in CS-B cells (Citterio et al., 1998) indicating that other properties of CSB also play a role during TCR. One such role could be the DNA wrapping activity of CSB, as this activity only requires CSB to bind ATP, not to hydrolyse it (Beerens et al., 2005). Alternatively, CSB may function as a structural factor in the physical stabilisation of repair complexes.

Several interactions of CSB with other factors involved in TCR and transcription have been observed. Most observations suggest an early role after RNA polymerase stalling, for example in recruitment of subsequent (repair) factors. Firstly, CSB binds directly to elongating RNA pol II (Tantin et al., 1997), an interaction that is stabilised following the induction of DNA damage (van den Boom et al., 2004). CSB also interacts with CSA (Henning et al., 1995), but CSA itself does not interact directly with RNA pol II (Tantin et al., 1997). Indeed, translocation of CSA to the nuclear matrix following DNA damage is dependent on CSB protein (Kamiuchi et al., 2002), as does the interaction of CSA with hyperphosphorylated (elongating) RNA pol II (M.I. Fousteri, pers. comm.). Finally, the *S. cerevisiae* homolog of CSB, Rad26, is involved in transcription past damaged bases independent from NER or BER, reinforcing the idea that CSB is intimately involved in ongoing transcription. These data also suggest that CSB is present at TCR sites before CSA and recruits, or helps recruit, CSA to a TCR site.

In addition, workers from the group of Cooper recently presented evidence that XPG when bound to a small DNA bubble (Dunand-Sauthier et al., 2005; Thorel et al., 2004) can interact with CSB, stimulating its ATPase activity (Sarker et al., 2005). These authors also showed that CSB together with XPG can interact with stalled RNA pol II ternary complexes (Sarker et al., 2005). Finally, the nuclease activity of XPG is inhibited in this situation but addition of TFIIH together with ATP relieves this inhibition (Sarker et al., 2005). Interestingly, a highly similar mechanism of NER endonuclease inhibition being relieved by TFIIH and ATP was observed before but in a simpler system using only a bubble DNA substrate, TFIIH, ATP and XPG or ERCC1-XPF (Winkler et al., 2001).

Much less is known about CSA. This 44 kDa protein lacks enzymatic activity; its main features are five WD repeats which may play a role in protein-protein interactions. CSA interacts with CSB and the p44 component of TFIIH (Henning et al., 1995). Moreover, CSA resides in a multi-subunit complex with the DDB1 protein (the large subunit of the UV-DDB damage-recognising heterodimer; see chapter 3.1), a ubiquitin-ligase activity in the form of cullin 4A and the COP9-signalosome (CSN) (Groisman et al., 2003). This complex associates constitutively with RNA pol IIa (the hypophosphorylated initiating form of RNA pol II) and after UV also interacts with RNA pol IIo (the hyperphosphorylated elongating form of RNA pol II). Also, after UV more CSN associates with the CSA complex, downregulating the ubiquitin-ligase activity (Groisman et al., 2003). However, the significance of these various associations and changing ubiquitin-ligase activity for TCR is as yet unknown.

XAB2 was only recently discovered as a protein implicated in TCR, transcription and pre-mRNA splicing (Nakatsu et al., 2000; Yonemasu et al., 2005). It consists mainly of 15 tetratricopeptide repeats. It interacts with XPA, CSA and CSB, and RNA pol II and its importance is underlined by the observation that mice lacking XAB2 suffer from pre-implantation lethality (Yonemasu et al., 2005). As yet, little more is known about XAB2.

2.2 Inherited disorders resulting from defective NER

The importance of the NER process at the organismal level is underlined by the existence of various rare autosomal recessive inherited disorders that are caused by mutations in proteins involved in NER (reviewed in Bootsma et al., 1998; Bootsma et al., 2001). The clinical appearance and a brief explanation of the most striking features of these diseases are given below.

Xeroderma pigmentosum (XP)
Patients suffering from XP display extreme sun-sensitivity, parchment skin (xeroderma) and freckles (pigmentosum) and a highly (>1000-fold) increased incidence of cancer in sun-exposed parts of the skin. Seven repair-deficient complementation groups can be distinguished (XP-A through XP-G), resulting from mutations in genes encoding NER proteins. Cells from all these complementation groups are compromised in GGR and with the exception of XP-C (Venema et al., 1991) and XP-E (Hwang et al., 1999) also in TCR. More severe cases of XP, mostly from complementation groups A and D also show mental retardation which probably arises from neuronal degradation following the accumulation of unrepaired damages in these cells. The observation that XP-C and XP-E are virtually free of neurological abnormalities suggests that removal by TCR of lesions from the transcribed strand of genes is critical for the development of neurological abnormalities and that functional TCR is sufficient to prevent these symptoms from occurring. When XP is diagnosed early, patients can be protected from sunlight which dramatically reduces their risk to develop skin tumours and accordingly increases their lifespan.

The eighth XP complementation group (XP variant or XP-V) remarkably is not deficient in NER, but bears a mutated DNA polymerase η, abrogating the possibility to replicate past CPD in an error-free manner (Masutani et al., 1999; see also chapter 1.2). Despite being NER-proficient, XP-V patients display a characteristic XP phenotype, including hypersensitivity of the skin to sunlight and a dramatic increase in sun-induced skin cancers.

Cockayne syndrome (CS)
CS is comprised of two complementation groups, CS-A and CS-B. At the cellular level CS is characterised by a lack of TCR but not GGR; moreover, CS cells do not display a recovery of RNA synthesis (RRS), i.e. they are unable to recover from the transcription inhibition that follows introduction of DNA damage, a phenomenon that has been related to the TCR defect (Mayne and Lehmann, 1982; Venema et al., 1990; see also chapter 4.1).

In clinical appearance, the most striking difference between CS and XP is the absence of a cancer predisposition in the former, despite the presence of a hypersensitivity of the skin to sunlight in both. Several non-mutually exclusive explanations exist for the cancer-free phenotype of CS. Firstly, since GGR is functioning normally DNA damages can be removed from the entire genome, thus preventing the development of cancer through unrepaired lesions (as a comparison, XP-C is TCR-proficient but shows a typical high skin cancer incidence). Secondly, it is hypothesised that apoptosis efficiently gets rid of cells that could transform into cancer cells (Ljungman and Zhang, 1996). Finally, CS patients may die before they can develop cancer; the mean age of death is around 12 years. Furthermore CS patients are usually diagnosed at an early age after which they are protected from sunlight, further decreasing their chances of developing skin cancers. Although a cancer predisposition is absent CS patients do display a wide variety of clinical symptoms that are conversely absent in typical XP, ranging from dwarfism and cachexia

to mental retardation. These symptoms are thought to ultimately result from accumulating stalled RNA polymerases on DNA lesions in the transcribed strand. Normally removed during TCR, these interfere with transcription in non-dividing cells and replication in dividing cells, eventually leading to cell death.

In addition to the 'classical' CS, CS can be associated with XP resulting from mutations in the *XPB*, *XPD* or *XPG* genes. XP/CS patients display a clinical and cellular phenotype that combines features from XP and CS. The clinical symptoms encompass the skin cancer predisposition and hyperpigmentation of XP patients and the mental retardation of CS patients, whereas at the cellular level, cells display impaired GGR and TCR together with the inability to recover RNA synthesis following DNA damage (van Hoffen et al., 1999).

Trichothiodystrophy (TTD)

A third disorder associated with defective NER is the photosensitive form of TTD. Its main clinical features are sometimes summarised in the acronym PIBIDS, i.e. photosensitivity, ichtyosis, sulphur-deficient brittle hair and nails (the most typical feature and used as a diagnostic), impaired intelligence, decreased fertility and short stature. Like CS, TTD is not characterised by a cancer predisposition.

A small number of TTD cases are the result of mutations in the *XPB* and *TTDA* genes, but the vast majority of TTD-causative mutations are in the *XPD* gene. TTD mutations destabilise the TFIIH complex resulting in the cellular hallmark of TTD, reduced levels of TFIIH (Botta et al., 2002; Vermeulen et al., 2000). This, due to the dual role of TFIIH (see chapter 3.4), also compromises NER.

It has been hypothesised that the specific TTD features, which also include β-thalassaemia (Viprakasit et al., 2001), result from the gradual decrease of transcription in terminally differentiated cells while still high levels of transcription are required to produce e.g. cysteine-rich matrix proteins (in hair shafts) or β-globin (in erythrocytes) (de Boer et al., 1998). Whereas under normal circumstances, resynthesis of TFIIH can compensate for its instability, in these situations the level of TFIIH may become limiting.

The combined transcription/repair defect in TTD patients together with the absence of a cancer predisposition conflicts with the hypothesis that CS is cancer-free because GGR is functioning normally, since within TTD both GGR and TCR are affected. To explain this apparent discrepancy, it has been speculated that certain transcriptional responses necessary to transform a cell into a tumour cell are absent (Berneburg et al., 2000). Another explanation may be provided by the observation that NER in TTD cells is cell cycle dependent. As a result, dividing TTD cells display efficient repair of 6-4PP, coupled with TCR of CPD, which together might be sufficient to prevent TTD patients from displaying an increased skin cancer phenotype (Riou et al., 2004). Finally, TTD patients like CS patients may simply not live long enough to develop cancer.

Like CS, also TTD can occur in combination with XP (Broughton et al., 2001), resulting from mutations in the *XPD* gene. The occurrence of such combined disease phenotypes highlights the complex interactions that exist in the cell between GGR, TCR and transcription, and simultaneously stresses the fact that especially mutations in *XPD* can give rise to a wide variety of clinical outcomes.

2.3 Sequential incorporation of factors into the GGR complex

An important question regarding NER *in vivo* is its organisation. NER proteins have to constantly search the genome for damages and after their detection damages have to be removed to prevent the obstruction of transcription or replication. To accomplish this, NER can be organised in at least two principally different ways. Firstly, most or all proteins might form a pre-assembled holocomplex that moves through the nucleus as a single entity, the so-called 'repairosome'. Alternatively, single proteins and/or small subcomplexes might be attracted to the lesion one at a time in a sequential manner.

The first indications for the existence of a repair holocomplex originated from studies on NER in *S. cerevisiae*, in which part of the entire NER machinery could be isolated as being associated with TFIIH (Svejstrup et al., 1995) and, according to a later report, with Rad14, the *S. cerevisiae* homolog of XPA (Rodriguez et al., 1998). In addition, around the same time a multitude of other NER factor associations and isolation of partial or incomplete NER complexes was reported, including: XPC and TFIIH (Drapkin et al., 1994; Mu et al., 1995), TFIIH and XPG (Iyer et al., 1996; Mu et al., 1995), XPG and RPA (He et al., 1995), RPA and XPA (He et al., 1995; Li et al., 1995; Matsuda et al., 1995), and XPA and ERCC1-XPF (Li et al., 1995; Nagai et al., 1995), creating links between all the core NER factors. Taken together these observations led to the hypothesis that also in humans NER could be organised as a repairosome. Indeed, He and Ingles upon mild lysis of HeLa cells reported the isolation of a repair holocomplex (He and Ingles, 1997). The complex not only contained preincision proteins such as XPA, RPA, TFIIH, ERCC1 and XPG, but also RF-C, PCNA and even the DNA polymerases δ and ε (He and Ingles, 1997). Consistent with the presence of these latter proteins, the complex exhibited NER activity up to and including DNA resynthesis (He and Ingles, 1997).

The major advantage of organisation into a holocomplex is that a lesion can be processed immediately after it is detected, but a repair holocomplex also presents specific problems. In the nucleus damages have to be processed in dense heterochromatin, where access to the lesions might be restricted for a large holocomplex. Furthermore, the sheer size of such a massive complex (~1-2 MDa) might render it rather inefficient – in other words, its movement through the nucleus might be very slow – so that timely removal of lesions would be compromised. Rather, to ensure efficient repair the (damaged) DNA would have to be recruited to the repairosome which would reside at a fixed place within the nucleus, i.e. the organisation of repair should much resemble the organisation of replication and transcription. A final point of concern is the fact that many proteins have functions outside NER, such as TFIIH in transcription and RPA in DNA replication and homologous recombination. Permanent incorporation of these proteins into a repair holocomplex might distract them from functioning in other pathways.

A fundamentally different way of organisation is to incorporate every protein sequentially into the 'emerging' NER complex. Interestingly, the first indications for this manner of organisation were also obtained by studying interactions of *S. cerevisiae* NER proteins with TFIIH (Guzder et al., 1996b). Guzder et al. suggested that the conclusions of Svejstrup et al. were likely to be caused by two experimental artefacts. First, two proteins reported to copurify with His-tagged TFIIH bound by themselves to the nickel column used by Svejstrup and co-workers (Guzder et al., 1996b). Second, Svejstrup et al. had reported that several proteins eluted in the same fraction of a size-exclusion column (Svejstrup et al., 1995), but the column in question was very likely to possess too little resolving power to be able to see the small differences in size between the various

NER proteins. Using a different size-exclusion column, Guzder et al. found the NER proteins to elute in different fractions (Guzder et al., 1996b). The findings of He and Ingles (1997) may not stand up to close scrutiny either. Firstly, these authors used the same size-exclusion column as Svejstrup and co-workers (Svejstrup et al., 1995). Secondly, their XPA-affinity column might actually detect separate individual interactions between proteins that do not actually reside in a large complex, e.g. XPA and TFIIH, XPA and ERCC1. A further discussion of the possibility of experimental artefacts in the reports of repair holocomplexes can be found in chapter 8 and in Araujo and Wood (1999).

Most problems associated with a repair holocomplex are circumvented by sequential assembly: single proteins and small subcomplexes can access the DNA in tightly packed chromatin, and will diffuse significantly faster than a holocomplex, together ensuring rapid screening of the entire genome. Also, since most proteins only interact strongly at the site of a lesion, they can function freely in other pathways than NER when no damages are present. Recently much evidence has been put forward supporting the latter model (Araujo et al., 2001; Hoogstraten et al., 2002; Houtsmuller et al., 1999; Mone et al., 2004; Riedl et al., 2003) and chapters 6 and 8 and the idea of a repairosome in human cells has been virtually abandoned.

A related question can be asked about the disassembly of the repair complex. Is it dismantled at once – together with or immediately after the release of the oligonucleotide, or during/by the repair replication step – or sequentially, similar to its assembly? Little research has been done on this matter; most authors either assume that all preincision proteins leave at once some time shortly after incision or step over the subject altogether, leaving a blank space between incision and resynthesis.

From a conceptual point of view it is conceivable that one or more proteins are released from the complex before repair is completed. The major advantage of this 'early release' would be that proteins quickly become available to initiate other repair events after having executed their function in the current one, leading to more effective repair. The only consistently reported early release is that of XPC-hHR23B leaving the NER complex upon the recruitment of XPG (Riedl et al., 2003; Tapias et al., 2004; Wakasugi and Sancar, 1998). In addition, Riedl et al. find that XPA and TFIIH leave the complex upon the arrival of ERCC1-XPF (Riedl et al., 2003). Measurements of the mobility of XPC-GFP in a live nucleus (Hoogstraten, submitted) suggest that XPC is attached to the NER complex significantly shorter, for approximately 2 minutes, than other factors such as ERCC1-XPF (Houtsmuller et al., 1999), TFIIH (Hoogstraten et al., 2002) and XPA (chapter 8) which are involved in NER for ~4-5 minutes. These latter findings on the one hand provide support for the notion that XPC leaves the complex before other NER proteins (although not necessarily directly following entry of XPG into the complex), but are ambiguous towards an early exit for TFIIH and XPA.

Kinetic modelling of the NER reaction confirms that an early release of XPC-hHR23B can give rise to enhanced repair rates or alternatively, lower the required concentration of XPC-hHR23B in the nucleus (Politi et al., 2005). This latter effect might be more important since XPC-hHR23B at higher concentrations is toxic to the cell (Ng et al., 2003; W. Vermeulen, pers. comm.). In this context it is also relevant to note that the nuclear concentration of XPC-hHR23B is lower than that of other investigated NER proteins (Araujo et al., 2001).

On the other hand, not all proteins necessarily dissociate immediately after dual incision: Riedl and co-workers found that no functional XPG was released following dual incision (Riedl et al., 2003). Although consistent with a possible role of XPG in the recruitment of late NER

factors, most notably PCNA (Gary et al., 1997), the fact that Riedl et al. did not measure release of XPG directly via Western blotting but indirectly via activity (incision) assays leaves the possibility open that XPG is released, but in an inactive form. Measurements of the mobility of XPG following UV irradiation indicate that XPG is bound to the complex for ~4 min, i.e. comparable to the binding time of other NER factors (except XPC) (D. Zotter, manuscript in preparation), suggesting that XPG is released simultaneously with the other NER factors (with the possible exception of XPC) following dual incision. In chapter 10, we address the possible release of factors from the NER complex utilising an *in vivo* competition assay consisting of a dual UV irradiation combined with the use of DNA synthesis inhibitors and NER-deficient cells. Our findings are consistent with a model in which all preincision NER proteins are released following dual incision, except RPA, which remains bound to assist in the DNA resynthesis stage. XPC may be released directly after incision, while the other preincision repair proteins stay associated with the lesion site and assist in the recruitment of the postincision factors (RF-C, PCNA, DNA polymerases and DNA ligase I).

2.4 NER substrate specificity: the bipartite recognition model

A striking feature of the GGR pathway of NER is its wide substrate specificity. GGR is capable of processing a large variety of lesions, ranging from the large chemical adducts formed by benzo[a]pyrene, via the small intrastrand crosslinks formed by cisplatin, to the purely structural (non-adduct) change of UV-induced photolesions. In contrast, processes such as BER or DSBR deal with a narrow set of lesions and repair by these processes is initiated by lesion-specific proteins. Although human MMR utilises one factor, hMSH2-hMSH6, to detect all possible single base pair mismatches, these mismatches (present in only a limited number of possibilities) all display a similar structure. The general structure of insertion/deletion loops, which are processed by the other MMR-initiating factor hMSH2-hMSH3, is also quite constant.

The prevailing model to explain the mechanism of damage recognition in NER is the 'bipartite' model put forward by workers from the lab of Naegeli, who found that a small mismatched bubble and a non-disturbing chemical modification to the DNA were only processed by NER when they were combined (Hess et al., 1997). The bipartite model thus suggests that NER probes the DNA for two types of changes to the normally stacked double helix. Firstly, NER processes (i.e. 'recognises') lesions that disturb this base stacking, usually giving rise to small bubble structure (Gunz et al., 1996; Hess et al., 1997). Secondly, NER will only proceed to the incision stage if concurrently with the helix distortion the chemical composition of the DNA is changed (Hess et al., 1997). Which proteins in the GGR pathway operate in which stage of this bipartite recognition is discussed in the next chapter.

Chapter 3

Global genome repair proteins and their functions

3 Global genome repair proteins and their functions

In Figure 1 (see Appendix), a model is given for the GGR pathway of NER, its various stages and the factors that are involved in every stage. In this chapter, these factors are highlighted in the order in which they are thought to play their role, and their functions in NER are elaborated on in detail. In addition, attention is given to the interplay between factors – which plays an important role in the multistep process that NER is.

3.1 UV-DDB: a key factor assisting damage recognition

Several proteins and protein complexes have been proposed to play a role in the damage recognition step of GGR: the UV-damaged DNA binding factor (UV-DDB) (Chu and Chang, 1988; Hwang et al., 1999; Tang et al., 2000), XPA (Jones and Wood, 1993; Robins et al., 1991), the complex of XPA and RPA (Asahina et al., 1994; Li et al., 1995; Mu et al., 1996; Wakasugi and Sancar, 1999), XPC-hHR23B (Reardon et al., 1996; Sugasawa et al., 1998) and RPA (Burns et al., 1996; Reardon and Sancar, 2002). Although the XPC-hHR23B complex is now generally considered the damage recognition factor for most lesions, there is considerable evidence that UV-DDB plays an important role in assisting repair of several types of lesions. First and foremost, GGR of CPD is strictly dependent on UV-DDB (Fitch et al., 2003b; Hwang et al., 1999; Tang et al., 2000). Additionally, our own research as described in chapter 9 has pointed out that also the repair of low levels of 6-4 photoproducts by GGR is strongly enhanced by (though not dependent on) UV-DDB.

UV-DDB is a heterodimer of the DDB2 and DDB1 proteins (formerly called p48 and p127 or p125, respectively) and was originally purified as a factor that bound specifically to UV-irradiated DNA (Feldberg and Grossman, 1976). Later, this factor was isolated from cell extracts as the only factor to readily bind UV- or cisplatin-damaged DNA and was found to be missing from a subset of cells assigned to the xeroderma pigmentosum group E (Chu and Chang, 1988). It has only recently become clear that in fact all cells from XP-E are deficient in UV-DDB activity (see below). Indeed, UV-DDB displays a high affinity for UV-induced photolesions, especially 6-4PP – as a matter of fact, the highest of all putative damage recognition factors (Batty et al., 2000) – while in addition, the complex exhibits a moderate affinity for several other types of lesions (Batty et al., 2000; Chu and Chang, 1988; Feldberg, 1980; Feldberg and Grossman, 1976; Feldberg et al., 1982; Fujiwara et al., 1999; Payne and Chu, 1994; Wittschieben et al., 2005). Nevertheless, its role in NER has been controversial from the moment of its discovery, due to several complicating issues.

The first complication was the misclassification of several cells in XP group E. Based on cell fusion/complementation experiments using unscheduled DNA synthesis (UDS) as read-out, these assays were complicated by the high UDS levels (50-80% of normal cells) displayed by XP-E cells. Recent re-evaluations combined with sequencing of the *DDB2* gene instead classified cells in groups such as XP-F and XP-V (Itoh and Linn, 2001; Itoh et al., 2000; Rapic-Otrin et al., 2003). Given these problems, nowadays XP-E is assigned based on the absence of the UV-DDB activity and on mutations in *DDB2*, instead of cell complementation studies (as suggested by Cleaver et al., 1999). It should be noted that all XP-E causative mutations are found in the

DDB2 gene, with no mutations known in *DDB1*. The reason for this is likely that DDB1 is an essential factor for development (Takata et al., 2004). Secondly, when UV-DDB has been included in *in vitro* NER reactions, contradictory findings have emerged, reporting that NER was either stimulated (Aboussekhra et al., 1995; Wakasugi et al., 2001), not effected (Araki et al., 2000; Rapic-Otrin et al., 1998) or even inhibited (Aboussekhra et al., 1995) [at high UV-DDB concentrations]; Kulaksiz et al., 2005) by UV-DDB. In any case, UV-DDB is not required for NER *in vitro*, leading to the suggestion that it might play a role in the repair of lesions within chromatin, rather than on naked templates (Rapic-Otrin et al., 1997). Unfortunately, its role in repair in the chromatin context is also a subject of dispute. Hara and co-workers measured excision of a single 6-4 photoproduct from a nucleosomal substrate and found rates to be comparable in extracts from HeLa cells, which contain functional UV-DDB, and from Chinese hamster ovary cells (Hara et al., 2000) expressing very low levels of UV-DDB (Hwang et al., 1998; Tang et al., 2000). The authors concluded that UV-DDB does not play a role in repair in chromatin at the nucleosomal level (Hara et al., 2000), but it cannot be excluded that the low level of UV-DDB present in these hamster cells was enough to stimulate repair, resulting in kinetics similar to HeLa cells. Alternatively, the procedure of making cell extracts from nuclei could abolish the effect UV-DDB has on NER in an intact nucleus, through the loss of some protein functions. It can also not be excluded that UV-DDB has an effect on higher levels of chromatin organisation than the nucleosome.

Finally, human XP-E and rodent cells, both of which are deficient in UV-DDB, show only a partial repair defect, i.e. only repair of CPD from non-transcribed DNA is absent. Although additionally in XP-E cells 6-4PP repair is mildly retarded compared to normal human cells at UV doses of 10 J/m^2 (Hwang et al., 1999) and 5 J/m^2 (chapter 9), at 30 J/m^2 repair in normal human and XP-E cells is indistinguishable (chapter 9). In contrast, 6-4PP repair in rodent cells is comparable to normal human cells; depending on the dose reaching completion within 4-8 hours following irradiation (Hwang et al., 1999; Tang et al., 2000 and chapter 9).

Taken together, these observations have led to the hypothesis that UV-DDB is not essential for damage recognition of all lesions but is necessary only for repair of those lesions that are otherwise poorly recognised by XPC-hHR23B, such as CPD (Hwang et al., 1999). In addition, our findings as described in chapter 9 suggest that UV-DDB may accelerate repair of lesions that are proper substrates for XPC-hHR23B, such as 6-4PP.

The role of CPD repair in cellular survival and mutagenesis

Interestingly, several types of normal rodent cells despite their lack of CPD repair show no reduced clonal survival following UV irradiation compared to normal human cells. This apparent discrepancy has been dubbed the 'rodent repairadox' (reviewed in Hanawalt, 2001). The CPD repair deficiency in rodent cells is partly thought to arise when rodent cells are grown in culture, as several observers have reported that repair of CPD declines when rodent cells are transferred from the skin to culture (Mullaart et al., 1988) and continues to decline following subsequent rounds of cell division (Ben-Ishai and Peleg, 1975; La Belle and Linn, 1984; Yagi, 1982). It appears that the underlying mechanism is the silencing by methylation of the *DDB2* gene (Hwang et al., 1998). At least in mouse cells, another factor contributing to the lower efficiency of CPD repair in rodent cells is the lack of transcriptional control over the *DDB2* gene by p53, leading to constitutively lower levels of UV-DDB and a lack of DNA damage-induced upregulation of DDB2 protein levels (Tan and Chu, 2002; see also chapter 3.3).

Although interesting, these observations do not explain the high clonal survival of cultured rodent cells. The main difference between cultured human and rodent cells concerning their UV-survival instead appears to be their response to DNA damage checkpoints. Human cells exhibit a p53-dependent apoptotic response to UV damage (Smith and Fornace, 1997; Ziegler et al., 1994) as do mouse cells *in vivo* (Tron et al., 1998) whereas in cultured rodent cells this response is evidently absent.

The similar clinical phenotype of XP-E patients compared to other XP groups implies that in XP-E CPD are the primary cause for the introduction of mutations and the formation of cancer. Functional TCR removes CPD from the transcribed strand of genes, ensuring that XP-E cells can proliferate without major problems, but the price is paid in the form of a high damage load carried over into S-phase, leading to the increased introduction of mutations. In other XP groups too, CPD may be the main causative lesion for the introduction of mutations, given its three-fold higher induction compared to 6-4 photoproducts.

Finally, mouse keratinocytes *in vivo* repair CPD as poorly as cultured mouse cells do (Mitchell et al., 1990; Qin et al., 1995), raising the question why mice do not display an XP-E-like cancer phenotype. The answer might simply be that mice, and other rodents, do not only possess a hairy fur but also live a nocturnal life; they may therefore not be exposed to substantial doses of UV in the wild. Additionally, mice only live for a few years, so they would not need to prevent the formation of tumours so vigorously as longer living animals such as humans do.

3.2 XPC-hHR23B

Recent *in vitro* and *in vivo* experiments provide evidence that the heterodimer consisting of XPC and hHR23B is the initial damage sensor in GGR: the complex plays a crucial role in the subsequent formation of the NER complex (Sugasawa et al., 1998 and chapter 6), predominantly via its interactions with TFIIH (Sugasawa et al., 1998; Uchida et al., 2002; Yokoi et al., 2000) and by opening of the helix slightly (Evans et al., 1997b; Tapias et al., 2004). Although *in vivo* this heterodimer is bound and further stabilised by centrin 2 (Araki et al., 2001; Nishi et al., 2005; see also below), centrin 2 is dispensable in *in vitro* reactions.

In vitro, XPC-hHR23B has an affinity for a broad spectrum of DNA lesions, including the UV-induced photolesions 6-4PP and CPD and cisplatin- and NA-AAF adducts (Batty et al., 2000; Reardon et al., 1996; Sugasawa et al., 1998). Additionally, XPC-hH23B binds to small bubbles devoid of a lesion (Sugasawa et al., 2001) and in fact, XPC-hHR23B displays quite a high affinity for undamaged DNA in general (Batty et al., 2000; Reardon et al., 1996). This characteristic is probably also responsible for the observation that *in vivo*, 90% of XPC-GFP in the nucleus is at any moment immobilised for short periods of time (a few seconds), most likely on DNA (D. Hoogstraten, manuscript submitted). Upon binding to DNA, XPC-hHR23B slightly opens the double helix (Sugasawa et al., 1998) and bends the DNA regardless of whether this is damaged or undamaged (Janicijevic et al., 2003). The resulting structure likely facilitates the recruitment of subsequent NER factors, most notably TFIIH (Sugasawa et al., 1998; Yokoi et al., 2000), XPA and RPA.

XPC and hHR23B perform distinct functions in the repair reaction. The 125 kDa XPC protein by itself can bind DNA and displays an affinity for damaged DNA. Furthermore *in vitro*, XPC can recruit TFIIH to damaged DNA (Yokoi et al., 2000) and function in NER (Reardon et

al., 1996; Sugasawa et al., 1996) without a requirement for hHR23B, while in mice *in vivo*, XPC can correct the (XP-C-like) repair defect in HR23-deficient cells (Ng et al., 2003). Thus, XPC is the subunit active in NER. In contrast, the 58 kDa hHR23B protein stimulates XPC-dependent repair in reconstituted NER *in vitro*, but does not allow NER in the absence of XPC (Araki et al., 2001; Reardon et al., 1996; Sugasawa et al., 1996), in line with the repair-deficiency observed in XP-C patients (who still express hHR23B). Instead of playing a direct role in repair, (h)HR23B protects XPC from proteasomal degradation (Araki et al., 2001; Ng et al., 2003), as does its *S. cerevisiae* homolog Rad23 with Rad4, the XPC homolog (Ortolan et al., 2004). XPC and hHR23B both reside in the nucleus (van der Spek et al., 1996) and almost all XPC is complexed with hHR23B (Masutani et al., 1994), but because of the abundance of hHR23B inversely only a fraction of hHR23B is complexed to XPC (Sugasawa et al., 1996; van der Spek et al., 1996). A small fraction of XPC is complexed with hHR23A, a close homolog of hHR23B (Araki et al., 2001); a mouse model showed that only in the absence of HR23B substantial amounts of XPC-HR23A are found (Ng et al., 2003). From the embryonic lethality of HR23A-HR23B double knockout mice (Ng et al., 2003), it can be inferred that these proteins serve essential functions outside NER that remain to be elucidated.

In mouse embryonic fibroblasts the first 6-8 hours post UV irradiation or post NA-AAF treatment, XPC is further stabilised in an HR23B-dependent manner resulting in a higher repair capacity (Ng et al., 2003) (the mechanism of this stabilisation is discussed in more detail in section 3.3). As mentioned above, constitutively high levels of XPC appear to be toxic to the cell (Ng et al., 2003; W. Vermeulen, pers. comm.), perhaps as a result of the affinity of XPC for normal dsDNA (Batty et al., 2000; Reardon et al., 1996) but more likely because of its high affinity for small bubble structures (Hey et al., 2002; Sugasawa et al., 2001). Such bubbles are routinely formed during transcription, so it is conceivable that XPC-HR23B will interfere with this process if present in too high concentrations. Therefore, it seems that under normal conditions (i.e. in the absence of DNA damage) XPC levels are kept low, and are post-translationally increased via stabilisation by HR23B only when necessary, i.e. when DNA damage is present.

XPC-hHR23B probes for helix destabilisation assisted by UV-DDB

The first step in the bipartite model for damage recognition in NER is probing for disturbed base stacking (see chapter 2.4). A DNA helix that is destabilised by a lesion is locally more flexible, making it easier for the DNA to bend and be bent. The NER factors with the highest affinity for damaged DNA, UV-DDB and XPC-hHR23B, both indeed bend damaged DNA upon binding (Fujiwara et al., 1999; Janicijevic et al., 2003). In addition, many DNA lesions themselves introduce a bend in DNA which will also facilitate binding of the damage-recognising proteins. For example, 6-4PP introduce a bend of ~40° in DNA, while also the base pairing is somewhat disturbed (Kim and Choi, 1995; Kim et al., 1995), and these lesions are good substrates for XPC-hHR23B and UV-DDB. In contrast, CPD only introduce an angle of 7-10° and retain the hydrogen bonds between the A and T bases (Kim and Choi, 1995; Kim et al., 1995) and consequently these lesions are poorly recognised by the XPC complex, which requires the assistance of UV-DDB to initiate their repair. Interestingly, the introduction of two T:G mismatched base pairs in the CPD does not disturb the hydrogen bonds, but does considerably increase the angle formed in the DNA (Lee et al., 2004) coinciding with an increased binding affinity by XPC-hHR23B (Sugasawa et al., 2001).

In a series of DNA binding and repair experiments, Sugasawa and co-workers showed that

XPC-hHR23B is capable of binding to small bubble structures distorting the DNA helix, regardless of whether these contain a lesion (Sugasawa et al., 2001; Sugasawa et al., 2002). Given the absence of base pairing within a small bubble (for example, a cisplatin adduct is sensitive to $KMnO_4$ approximately 3 nt on either side of the lesion; Constantinou et al., 1999; Evans et al., 1997a; Tapias et al., 2004) it is likely that also these structures are easily bent upon binding of XPC-hHR23B. *In sum*, XPC-hHR23B appears to be responsible for sensing destabilisations in the DNA helix. The mechanism of action of UV-DDB is less well-characterised, but UV-DDB might detect destabilisations in a comparable manner. Although only essential for the repair of CPD, it seems likely that UV-DDB assists in the repair of several other types of lesions similarly to its stimulation of 6-4 photoproduct repair, i.e. at physiologically relevant low doses. Meanwhile, dual incision only occurs when a lesion is present within the bubble (or more general, the region of destabilised DNA) bound by XPC-hHR23B (Sugasawa et al., 2001). XPA or XPA in conjunction with RPA are likely to be responsible for this second, damage verification step of NER, possibly in cooperation with TFIIH (see chapters 3.4 and 3.5).

3.3 Post-transcriptional regulation of UV-DDB and XPC – regulating the GGR pathway via the damage-recognition step

Degradation of DDB2 following UV

The DDB2 subunit of UV-DDB tightly binds to UV-damaged chromatin, after which it is consumed by proteasome-mediated degradation (Chen et al., 2001; Fitch et al., 2003b; Rapic-Otrin et al., 2002). After this initial degradation, DDB2 levels are restored to their original levels within the next 24 hours, with an overshoot at 48 hours after UV, likely caused by the p53-dependent transcriptional activation of the *DDB2* gene (Hwang et al., 1999; Liu et al., 2000; Nichols et al., 2000). Recently, the molecular mechanism of DDB2 degradation has been clarified. Cullin 4A, an E3 ubiquitin-protein ligase, coimmunoprecipitates with UV-DDB (Shiyanov et al., 1999). This interaction results in the ubiquitination of DDB2, causing it to be degraded (Chen et al., 2001; Nag et al., 2001). Degradation of DDB2 in turn results in a decrease in UV-DDB damaged DNA binding activity (Chen et al., 2001). The hypothesis that follows from these observations, i.e. that cullin 4A is responsible for the observed degradation of UV-DDB following UV irradiation, has since been confirmed (Matsuda et al., 2005; Rapic-Otrin et al., 2002; Sugasawa et al., 2005).

Given the high affinity of UV-DDB for especially 6-4PP, it is conceivable that the ubiquitination and/or degradation of UV-DDB is necessary to allow its removal from this type of lesion before the subsequent action of other NER factors can take place. This view is supported by several observations: (i) degradation of UV-DDB is observed in NER-deficient cells as well – most relevantly including XP-C (Rapic-Otrin et al., 2002); (ii) the kinetics of 6-4PP repair and the degradation of DDB2 are highly similar, both occurring primarily in the first few hours after UV irradiation (Fitch et al., 2003b; Liu et al., 2000; Nichols et al., 2000; Rapic-Otrin et al., 2002); and (iii) ubiquitination of UV-DDB dramatically reduces its DNA binding activity (Sugasawa et al., 2005). It would be interesting to see whether mutations in DDB2 that prevent its ubiquitination give rise to a dominant negative effect on repair (of 6-4PP); the existence of a patient expressing a truncated DDB2 with a specific defect in 6-4PP repair but not CPD repair (Itoh et al., 1999) might be especially significant in this matter.

The known interactions between cullin 4A and UV-DDB were extended by Groisman and co-workers (Groisman et al., 2003). Two complexes of almost identical composition are present in the cell, each containing the DDB1 subunit of UV-DDB, the cullin 4A-based ubiquitin-ligase activity, the COP9-signalosome (CSN), which is a regulator of cullin-based ubiquitin-ligase activity, and either DDB2 or CSA but not both (Groisman et al., 2003). The DDB2-containing complex when engaged in GGR (after UV irradiation), loses its CSN and thus likely gains ubiquitin-ligase activity (Groisman et al., 2003). In contrast, the CSA-containing complex gained more CSN following UV irradiation, further downregulating this activity (Groisman et al., 2003). The implications of this latter phenomenon for TCR are not clear yet.

It is interesting to note that DDB2 is degraded to virtual absence during repair of a lesion for which it is not strictly required, i.e. 6-4 photoproducts, thus rendering it unavailable for repair of CPD, which do require DDB2. The most likely explanation seems that the observed depletion of DDB2 is an experimental artefact due to the single high dose of UV light used in laboratory experiments, a situation that would rarely if ever occur in nature. Rather, an all-day long continuous exposure to a low UV flux might lead to a balanced production and degradation of DDB2 – sufficient to cope with the rate of photolesion induction and resulting in a more or less equilibrated amount of DDB2. Given the periodicity and regularity of the induction of UV damage to the DNA it is also not surprising to find an upregulation of *DDB2* expression 48 hours following UV when, under laboratory conditions, 'all damages have been removed'. In nature, it is highly likely that 48 hours following the introduction of UV damage, UV damage will again be introduced – the induction can thus be considered an adaptation process (also suggested in McKay et al., 2005).

Increased activity of XPC following UV

The finding that the UV-DDB-cullin 4A complex probably increases its activity when participating in repair is particularly interesting in the light of the second role that ubiquitination plays in NER: the monoubiquitination of XPC following the infliction of NER-types of DNA damage (Lommel et al., 2002; Ortolan et al., 2004; Sugasawa et al., 2005; Wang et al., 2005). In contrast to the ubiquitination of DDB2, ubiquitination of XPC does not result in its proteasomal degradation, because XPC is protected from degradation by its heterodimeric partner, HR23 (Araki et al., 2001; Ng et al., 2003; Ortolan et al., 2004). HR23B contains two ubiquitin-associated (UbA) domains that interact with ubiquitin. Thus, the sequence of events that occur following DNA damage is likely to be: XPC is monoubiquitinated, which leads to an enhanced interaction with HR23B; the resulting protection against degradation in turn increases overall levels of XPC, leading to the higher repair capacity of cells observed in the first hours after UV (Ng et al., 2003). Simultaneously, HR23 can prevent polyubiquitination of its partners thereby also precluding their proteasomal degradation (Ortolan et al., 2000).

Not only does ubiquitination of XPC prevent its degradation, it also increases the affinity of XPC for DNA in general, regardless of whether this is damaged or not (Sugasawa et al., 2005); moreover the ubiquitination is reversible, upon which the affinity of XPC for DNA decreases accordingly (Sugasawa et al., 2005). Remarkably, but in the light of the above maybe not surprisingly, ubiquitination of XPC is executed by the UV-DDB-cullin 4A complex (Sugasawa et al., 2005). Together, these findings result in a model for the early stage of GGR in which ubiquitination of the main damage recognition complexes plays an important role in their loading and unloading on the DNA. The model is depicted in Figure 2 (see Appendix). Adjusting the level of XPC post-transcriptionally allows the cell to rapidly regulate its GGR capacity, avoiding

the slower process of upregulation via transcription. Although XPC is actually transcriptionally upregulated following DNA damage (by p53), this upregulation occurs at a time when TCR is expected to have removed most lesions from transcribed DNA; see below). Similarly to DDB2 (see above), upregulation of XPC could prepare the cell for a 'second round' of DNA damage, e.g. exposure to UV light from the sun one day later.

Recently it was reported that SUMOylation of XPC following UV irradiation also can protect XPC from degradation (Wang et al., 2005). Although dependent on UV-DDB (like its ubiquitination), the co-dependence on XPA protein (Wang et al., 2005) could suggest that this modification occurs in a later stage of the NER reaction, when the complex is (nearly) completely formed.

Other XPC-regulating proteins

The levels of XPC are modulated by two more proteins. The small centromere protein centrin 2 (18kDa) assists in the stabilisation of XPC by hHR23B (Araki et al., 2001; Nishi et al., 2005). Recently it was found that centrin 2 stimulates NER both *in vitro* and *in vivo* via its interaction with XPC (Nishi et al., 2005). The established function of centrin 2 lies in centrosome duplication, suggesting a coupling between NER and replication, but evidence for such a coupling has yet to be reported.

Finally, the unusual protein kinase C (PKC) ζ influences XPC and hHR23B at the post-translational level. Cells overexpressing PKCζ show increased resistance to genotoxic agents that induce lesions repaired by NER, such as UVC and cisplatin, and display concurrent increased repair rates of the respective lesions (Louat et al., 2004). These effects appear to be a direct consequence of a 10- and 5-fold increase in levels of XPC and hHR23B protein, respectively, while their mRNA levels are unaffected (Louat et al., 2004). A putative role for PKCζ in stabilising XPC fits well with similar findings concerning other members of the PKC family, which regulate various proteins involved in genomic stability such as DNA-PKcs (Bharti et al., 1998), hMSH2 and hMSH6 (Christmann et al., 2002; Humbert et al., 2002) and MGMT (Srivenugopal et al., 2000).

Regulation at the transcriptional level: a supervising role for p53

The tumour suppressor protein p53 is involved in NER through its regulation of DDB2 (Hwang et al., 1999) and XPC (Adimoolam and Ford, 2002) at the transcriptional level (reviewed in Ford, 2005). In p53-proficient cells, transcription of the *DDB2* gene is upregulated in a p53-dependent manner following DNA damage, resulting in an increased UV-DDB activity (Hwang et al., 1999). Also XPC is induced both at the protein and the mRNA level in a p53- and DNA damage-dependent manner (Adimoolam and Ford, 2002). Additionally, in p53-deficient cells the basal mRNA levels of the respective proteins are severely reduced compared to that in the p53-proficient counterparts (Adimoolam and Ford, 2002; Hwang et al., 1999). These results explain the earlier observation that efficient GGR of CPD requires the activation of p53 (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997), since both XPC and UV-DDB are essential for repair of CPD via GGR, and could also provide a rationale for the reduced repair of, and sensitivity to, the bulky anti-benzo[a]pyrene-diol-epoxide adduct observed in p53-/- cells (Wani et al., 2002; Wani et al., 2000). Finally, as mentioned in chapter 3.1, in mice the *DDB2* gene is not transcriptionally controlled by p53, partially explaining the lack of CPD repair in mouse (and possibly other rodent) cells (Tan and Chu, 2002).

3.4 TFIIH

TFIIH is a highly versatile protein, being involved in at least five (at last count) distinct cellular processes: RNA pol II transcription initiation (Flores et al., 1992; Gerard et al., 1991); RNA pol I transcription (Iben et al., 2002); global genomic NER (Drapkin et al., 1994; Schaeffer et al., 1993; van Vuuren et al., 1994; Wang et al., 1994); transcription-coupled repair (van Hoffen et al., 1999); and promoter-specific transactivation (Drane et al., 2004; Keriel et al., 2002; Liu et al., 2001). Furthermore, its CAK subcomplex contains a cyclin dependent kinase activity functioning in cell-cycle progression (Fisher and Morgan, 1994).

TFIIH is the largest factor in NER, both in number of subunits (ten) and in molecular weight (~450 kDa). Its subunits are XPB/p89, XPD/p80, p62, p52, p44, p34, the three subunits of the CAK subcomplex cdk7 (41 kDa), cyclin H (38 kDa) and MAT1 (32 kDa) and the 8 kDa small p8/TTDA, which was discovered only recently (Giglia-Mari et al., 2004; Ranish et al., 2004). There is some unclarity as to whether TFIIH is present in the cell as a complex harbouring all ten subunits ('holo-TFIIH') or whether several smaller subcomplexes exist beside holo-TFIIH.

Although holo-TFIIH can always be isolated from human cells, several early reports indicate the existence of other possible compositions for TFIIH. A 'core' has been found, consisting of XPB, XPD, p62, p52, p44 and p34, separate from the CAK complex (Rossignol et al., 1997); additionally XPD has been observed to complex with CAK, while the remaining factors constituted another complex (Drapkin et al., 1996; Reardon et al., 1996; Rossignol et al., 1997). In support of these findings it was found that (i) six-subunit core was functional in NER *in vitro* (Araujo et al., 2000; Mu et al., 1996) and (ii) several complexes, i.e. holo-TFIIH, the six-factor core and the smaller core (i.e., lacking XPD) were all functioning in transcription initiation *in vitro* (Tirode et al., 1999).

Other observations, on the other hand, indicate that in the nucleus holo-TFIIH is the predominant form, performing NER – and probably also all other functions of TFIIH. Firstly, as early as 1994 it was reported that microinjection of cells with an antibody against cdk7 led to an inhibition of NER (Roy et al., 1994). Secondly, pulldown of TFIIH with an antibody against cdk7 precipitated exclusively holo-TFIIH, with no TFIIH (measured by the presence of XPB and p62) remaining in the supernatant (Araujo et al., 2001). Thirdly, after local UV irradiation and immunofluorescent labelling, components of the CAK subunit localise at the site of local UV damage (Coin et al., 2006; M. Volker, unpublished observation). In short, there is an apparent contradiction between the supposed existence of several separate subcomplexes and reports suggesting that TFIIH is mainly present in holo-form in the cell. To add to the unclarity, in *S. cerevisiae* altogether different subcomplexes of TFIIH have been found, e.g. 'core' TFIIH comprises the homologs of XPD, p62, p52, p44 and MAT1, but not XPB and p34 (Chang and Kornberg, 2000). The lack of consistency between the various reports has led to the hypothesis that the observed variation in composition of TFIIH merely reflects differences in the various purification procedures and that in the cell holo-TFIIH is the predominant form of TFIIH (Araujo et al., 2001). If so, the question arises if and how CAK dissociates from core TFIIH to perform its function in the cell cycle; possibly, CAK is present in excess over core TFIIH.

Several enzymatic activities reside in TFIIH: XPB and XPD are DNA-dependent ATPases/DNA helicases (Roy et al., 1994; Schaeffer et al., 1994; Schaeffer et al., 1993; Sung et al., 1993; Weber et al., 1990), while cdk7 possesses kinase activity. The 3' to 5' helicase activity of XPB is essential in both NER and transcription initiation (Evans et al., 1997b; Guzder et al., 1994; Qiu et al., 1993; Sung et al., 1996); in contrast the 5' to 3' XPD helicase activity is necessary for NER

but dispensable for transcription initiation, where only the structural presence of XPD is required (Coin et al., 1999; Sung et al., 1988; Winkler et al., 2000). Still, functional XPD does enhance transcription when associated with the five-subunit 'small core' TFIIH (Tirode et al., 1999). In both NER and transcription TFIIH opens up a 10-12 nt stretch of helical DNA to form a single-strand bubble (Evans et al., 1997b; Holstege et al., 1996; Tapias et al., 2004). This structure might stabilise the binding between TFIIH and DNA, and in repair thus facilitate the further recruitment of NER factors to the NER complex via their interactions with TFIIH (Araujo et al., 2001; Evans et al., 1997b). The kinase activity residing in the CAK subcomplex targets different proteins depending on its associations. Association of CAK with core TFIIH confers a preference to phosphorylate the C-terminal domain of RNA pol II, stimulating promoter clearance and the start of transcription elongation (Rossignol et al., 1997; Tirode et al., 1999). On the other hand, CAK on it own phosphorylates cdk1, cdk2 and cdk4 (Nigg, 1996; Rossignol et al., 1997), promoting cell cycle progression (Fisher and Morgan, 1994). Finally, the yeast homolog of TFIIH was recently found to possess a ubiquitin-ligase activity residing in its Ssl1 subunit (the homolog of p44) (Takagi et al., 2005). This activity is involved in a transcriptional response and is not required for repair activity (Takagi et al., 2005). As yet its targets are unknown, but a likely possibility are transcriptional activators, for instance of DNA repair genes. It will be intersting to see whether the mammalian p44 subunit possesses a similar activity.

Additionally, most subunits of TFIIH perform specific non-enzymatic functions. The C-terminal domain of XPB stimulates incision by ERCC1-XPF and deletion of this domain, whilst not interfering with the structure or helicase activity of XPB nevertheless results in abrogated 5' NER incision (Coin et al., 2004; Evans et al., 1997b). Curiously, a direct interaction between ERCC1-XPF and the C-terminus of XPB has yet to be reported. XPD is the subunit that anchors CAK to TFIIH (Coin et al., 1999; Drapkin et al., 1996; Rossignol et al., 1997). The p62 subunit contains a PH domain that interacts with XPG (Gervais et al., 2004). Deletion of this domain resulted in an abolished *in vitro* NER activity of TFIIH without apparently affecting its transcriptional activity or its structure (Gervais et al., 2004). If *in vivo* mutations in or the deletion of this domain would have an analogous effect, it raises the intriguing question why there is no XP complementation group with mutated p62. The p52 subunit stimulates the helicase activity of XPB through a direct interaction (Jawhari et al., 2002); in a similar manner, XPD helicase activity is stimulated by the p44 subunit (Coin et al., 1998). Finally p8/TTDA functions as a stabilising factor (Coin et al., 2006; Giglia-Mari et al., 2004; Ranish et al., 2004). p8/TTDA furthermore stimulates the helicase activity of XPB (Coin et al., 2006) although apparently p8/TTDA and XPB do not interact directly (Coin et al., 2006). p8/TTDA does interact with XPD and with p52 (Coin et al., 2006); perhaps p8/TTDA thus stimulates XPB indirectly.

Possible roles for TFIIH in detecting chemical alterations and in regulating dual incision

Apart from helix unwinding TFIIH might perform other functions in NER. Rad3 is the *S. cerevisiae* homolog of XPD and is able to bind to a 3'-single-strand extension of dsDNA and from there can unwind duplex DNA in the 5' to 3' direction (Sung et al., 1987). This helicase activity can be blocked by damages in the strand to which Rad3 binds, while damages in the other strand pose no obstacle for unwinding (Naegeli et al., 1992; Naegeli et al., 1993). This strand-specific block may thus serve as both a damage sensor and as a strand discrimination signal (to determine in which strand of a disturbed DNA duplex the damage resides), which led to the hypothesis that this mechanism could contribute to damage recognition by NER (Naegeli et al., 1992; Naegeli et al.,

1993). The structure formed after the blocking of the helicase stabilises the binding between TFIIH and the DNA (Naegeli et al., 1992; Naegeli et al., 1993), and thus facilitates the further recruitment of NER factors to the NER complex via their interactions with TFIIH. When the helicases are not blocked by a lesion, the complex may disassemble (Wood, 1999), precluding gratuitous repair.

A recent finding by the group of Egly adds another layer of complexity to the damage recognition step of NER. Coin et al. reported that phosphorylation of XPB at lysine 751 leads to a specific inhibition of XPB activity in NER but not in transcription (Coin et al., 2004). This phosphorylation specifically inhibits the 5'-incision by ERCC1-XPF by preventing its recruitment to the NER complex, without affecting either the helicase or the transcriptional activity of XPB. Although these findings provide evidence suggestive of another regulatory mechanism in NER, further research is required to resolve some important questions that remain, including: is the phosphorylation status indeed actively changed *in vivo* after the induction of DNA damage, as their findings suggest; does the phosphorylation occur in XP(-C) mutant cells; and what proportion of total XPB is phosphorylated and what is the time course of phosphorylation and dephosphorylation. It will be interesting to observe whether cells expressing a mutated XPB incapable of phosphorylating lysine 751 show a distinct phenotype besides impaired NER.

3.5 XPA

As explained above, initially it was suggested that the 30 kDa XPA protein was directly involved in the first step of NER: damage recognition. The reason for this was twofold; firstly, XP-C cells only show compromised GGR whereas in XP-A cells also TCR is affected. Secondly, early biochemical characterisations showed that purified XPA has an increased affinity for damaged over undamaged DNA (Jones and Wood, 1993; Robins et al., 1991). These observations led to models in which XPA was thought to recognise all lesions after which XPC would be responsible for initiating the GGR pathway, for instance by stabilising an open intermediate complex that in TCR would be stabilised by RNA pol II (Mu et al., 1996; Reardon et al., 1996; van der Spek et al., 1996).

Shortly hereafter, it was found that XPA interacts with RPA (He et al., 1995; Lee et al., 1995; Li et al., 1995; Matsuda et al., 1995) and the resulting complex displayed a higher affinity for damaged DNA than XPA alone (Li et al., 1995). The XPA-RPA complex thus seemed a likely candidate for global damage recognition. Wakasugi and Sancar performed DNA binding and repair assays on an oligonucleotide harbouring a single 6-4 photoproduct. They found that damage removal was faster when XPA and RPA were the first factors to bind to DNA compared to when XPC was the first protein presented to DNA, and they concluded that RPA and XPA are the initial damage sensing factors of human NER (Wakasugi and Sancar, 1999).

On the other hand, evidence has accumulated supporting the view that XPC-hHR23B is the damage recognition factor for GGR. Using NA-AAF-treated adducted plasmids in an *in vitro* damage recognition-competition assay Sugasawa and co-workers showed that XPC-hHR23B is likely the damage detector that initiates NER *in vitro* (Sugasawa et al., 1998), while we have shown that *in vivo*, recruitment of XPC to UV lesions is independent of functional XPA whereas vice versa XPA nor RPA in the absence of XPC relocalise to UV lesions (chapters 6 and 8). The difference in the *in vitro* findings has been attributed partly to the shorter probe fragments used by Sancar's laboratory, but mainly to the lack of competitor DNA in their experiments (Batty et al., 2000). Further *in vitro* experiments by the group of Egly have corroborated the findings

by Sugasawa et al. and additionally have indicated that XPA is not incorporated into the NER complex until after the recruitment of TFIIH (Riedl et al., 2003). Although from our experiments described in chapter 6 it is technically not possible to exclude that *in vivo* XPA is actually recruited to the complex before TFIIH, Coin and co-workers recently observed that in TTD-A cells, which have a defect in helix opening during NER, TFIIH is recruited to sites of local UV but XPA is not (Coin et al., 2006), corroborating the *in vitro* data and further strengthening the notion that XPC and TFIIH are the first two factors to be involved in NER. Damage recognition in TCR, meanwhile, has become thought of as being triggered by stalling of the transcribing RNA polymerase at a lesion (originally proposed by Mellon and Hanawalt, 1989; Mellon et al., 1987), obviating the need for a dedicated damage-recognising protein in TCR.

Several reports by the group of Naegeli have indicated that XPA specifically binds to bent DNA duplexes, a feature that is incompatible with a function in direct lesion recognition but is characteristic of architectural proteins (Missura et al., 2001; Camenisch et al., 2006). As mentioned above, such bending of DNA is introduced upon binding of the damage-recognition proteins UV-DDB and XPC-hHR23B. During this binding XPA does not directly interact with (or 'recognise') the lesion, instead performing an 'indirect readout', in agreement with the wide substrate specificity of NER.

Damage verification by XPA: regulation of the endonucleases in cooperation with TFIIH?
As mentioned above, XPC-hHR23B has a high affinity even for undamaged DNA, and is frequently immobilised for short periods of time in the nucleus, most likely to DNA (D. Hoogstraten, submitted). XPC-hHR23B in turn interacts quite strongly with TFIIH (Araujo et al., 2001; Drapkin et al., 1994; Yokoi et al., 2000), so 'proto-NER complexes' consisting of XPC-hHR23B and TFIIH bound to DNA probably form regularly. It is important that these erroneous proto-NER complexes do not reach the incision stage, to prevent numerous strand breaks from being introduced into the genome. XPA is likely to play an important role in the second part of the bipartite damage recognition by NER, i.e. detection of the chemical modification of the DNA – often referred to as 'damage verification' (Missura et al., 2001; reviewed in Thoma and Vasquez, 2003). XPA might execute this damage verification in several different ways.

Firstly, binding of XPA to a lesion site could (further) stabilise a bona fide NER complex. If no lesion is present the complex is not stabilised by XPA and disassembles. As shown in chapter 10, the partial NER complex formed in XP-A cells following UV irradiation appears to be stable, seemingly arguing against this mechanism. In this case however, there are lesions present in the partial complexes which is likely to increase their stability (see chapters 3.2 and 3.4).

In addition to further stabilising an NER complex when a lesion is present, several observations indicate that XPA might perform a crucial role in directly and indirectly regulating the endonuclease activities, providing the second line of defence against aberrant cutting. First, the presence of XPA is required to allow recruitment of ERCC1-XPF (Li et al., 1995 and chapter 6). Also the incorporation of XPG into the NER complex has been reported to depend on XPA *in vitro* (Riedl et al., 2003), although *in vivo* this is not observed (chapter 6). Second, XPA might stimulate or even permit incision by XPG and ERCC1-XPF. This stimulation (or permission) could be an indirect effect that depends on the function of the TFIIH helicases. The mechanism can be inferred from two observations. Firstly, the presence of XPA specifically stimulates ATP hydrolysis activity of the TFIIH helicase subunits in the presence of a DNA lesion (Winkler et al., 2001). Addition of RPA further increases this stimulation (Winkler et al., 2001). Secondly,

this ATP hydrolysis is required to stimulate dual incision (Mu et al., 1996; Svoboda et al., 1993). Thus, XPA could be essential for endonuclease activity in the presence of a lesion. Inversely, if XPA by virtue of its affinity for damaged DNA is not able to detect a lesion within the complex, it will not associate with the NER complex, preventing the incision step.

Supporting a mechanism in which XPA stimulates the endonucleases either directly or indirectly is the observation that in XPA-deficient cells XPG is incorporated into the complex (chapter 6) but does not incise (Evans et al., 1997a). In contrast, in XPF-deficient cells both XPA and XPG are incorporated into the complex (chapter 6). In this situation, some authors have found that XPG is capable of making an incision (Evans et al., 1997a); others found that the presence of ERCC1-XPF is required before XPG can incise (Tapias et al., 2004). Despite this discrepancy, in both cases XPA was absolutely required for incision by XPG. Also, the presence of p8/TTDA which stimulates XPB helicase activity and promotes open complex formation (Coin et al., 2006) is required for XPA relocalisation to sites of UV damage; concurrently p8/TTDA not only promotes 5' incision by ERCC1 but also 3' uncoupled incision by XPG (Coin et al., 2006). The latter could be a direct effect of the stimulated open complex formation, an indirect effect of XPA recruitment to the complex, or (maybe most likely) a combination of both.

Taking all data together, XPA is not a principle damage recognition factor but instead a damage verification (or demarcation) protein in a later stage of NER, functioning to ascertain that the DNA covered by the NER complex indeed harbours a lesion (Missura et al., 2001; Sugasawa et al., 2001). It is noteworthy that the proposed roles for TFIIH and XPA in this stage of NER are very similar in nature, overlap to a large extent, and could likely occur in concert or even in synergy. Additionally, RPA is also likely to assist in this stage of NER (see below).

3.6 RPA

The RPA protein is a heterotrimer consisting of a large (70 kDa), middle (34 kDa) and small (17 kDa) subunit that displays an extraordinarily high affinity for single-strand DNA. Many DNA metabolic processes that involve ssDNA intermediates, including replication, recombination and various repair pathways, utilise RPA. Covering the ssDNA, RPA protects it from attacks by endonucleases and prevents the formation of unwanted secondary structures.

RPA is able to bind to ssDNA in two modes. In the one mode, it binds to a stretch of 8-10 nucleotides; in the other, far more stable (~100 fold) mode it binds 30 nucleotides (Blackwell and Borowiec, 1994; Kim et al., 1994; Kim et al., 1992). The stretches of ssDNA formed by the helicase action of TFIIH are of essentially the correct length for RPA to bind in 8-10 nt mode. After binding to this bubble, RPA might change to the 30 nt conformation, thereby creating stretches of ssDNA similar in length to the oligomer released after dual incision. Apart from playing a role in ssDNA protection, RPA is absolutely required for dual incision (Coverley et al., 1991). Interestingly, RPA is important for the proper positioning and stimulation of the NER endonucleases, XPG and ERCC1-XPF (de Laat et al., 1998b; He et al., 1996; Matsunaga et al., 1996). RPA binds with a specific polarity to the undamaged strand during NER. From this position it coordinates the binding of and incision by XPG and ERCC1-XPF. At its 3' end, RPA interacts with and stimulates activity of ERCC1-XPF in the opposing strand, resulting in the incision 5' to the lesion. Conversely, at its 5' end it allows the binding of XPG which can then incise 3' to the lesion (de Laat et al., 1998b).

RPA is also an essential component in the DNA resynthesis step of NER (see chapter 3.10). Thus, it is the only protein that is involved in both the pre- and post-incision stages of NER. The possibility that RPA remains localised to the undamaged DNA strand after excision of the damaged oligo, and possible consequences of its 'dual role' for the formation of the preincision NER complex are discussed in chapter 10.

RPA complements XPA in damage verification

Although it has been suggested that RPA (either alone or in combination with XPA) is implicated in the damage recognition step by virtue of a preference for cisplatin- (Clugston et al., 1992) and UV-damaged DNA (Burns et al., 1996) over undamaged DNA, or by direct interaction with a psoralen lesion in the absence of other repair factors (Reardon and Sancar, 2002), there is no support for these claims within the context of what else is known about the NER reaction. Our experiments in fact show that RPA does not translocate to sites of UV damage in XP-C cells (chapter 8) providing evidence against RPA as the initial damage recognizing factor. The binding of RPA to damaged DNA may actually reflect its role in damage verification, where it cooperates with XPA. While XPA detects a kink in the DNA (see above), RPA specifically binds to the unwound single-strand DNA at the site of the lesion (Missura et al., 2001). If either the kink or the single-strand are absent from the DNA, XPA or RPA respectively will not bind to the NER complex, in turn abrogating incision by the endonucleases.

Recently it has become doubtful whether XPA and RPA actually coexist in a complex in the nucleus as was originally thought (He et al., 1995; Lee et al., 1995; Li et al., 1995; Matsuda et al., 1995). *In vivo* diffusion measurements of XPA-GFP provided no evidence for an interaction of XPA with (the much larger) RPA, and in XP-A cells RPA readily relocalises to sites of subnuclear UV irradiation (chapter 8). These results suggest that both proteins normally only interact with each other in the context of an NER complex.

Taking above data together, it appears that XPA and RPA cooperate in the damage verification step of NER. Both play distinct, complementary and essential roles in the recruitment, positioning and activation of the endonucleases XPG and ERCC1-XPF, providing a double-check mechanism that will not allow dual incision to take place unless it is clear that a damage is present in the initial complex formed by XPC-hHR23B and TFIIH. As speculated above, TFIIH could provide further assistence in this step via its kinetic proofreading mechanism (chapter 3.4).

3.7 XPG

The 133 kDa XPG protein is a structure-specific endonuclease of the FEN-1 family (Liever 1997). It cuts specifically at or near the junction between the 3' end of single-stranded DNA and the 5' end of double-stranded DNA. In NER, XPG incises the damage-containing strand at a distance of 2-8 nt 3' of the lesion (Matsunaga et al., 1995; O'Donovan et al., 1994a; O'Donovan et al., 1994b).

Interactions between XPG, several subunits of TFIIH, and CSB have been observed (Araujo et al., 2001; Gervais et al., 2004; Iyer et al., 1996; Mu et al., 1995), although some investigators did not detect interactions between CSB and XPG (van Gool et al., 1997). XPG, TFIIH, CSB and RNA pol I were later found to reside in a complex that promotes RNA pol I synthesis (Bradsher et al., 2002), providing a rationale for the interactions described in previous reports.

Alternatively or additionally, the interactions between XPG, TFIIH and CSB in undamaged cells may reflect interactions that are important in the formation of a protein complex active in TCR (chapter 2.1), much like the interaction between XPC and TFIIH in undamaged cells (Araujo et al., 2000) reflects their concerted action in early steps of GGR (Yokoi et al., 2000).

Apart from its endonuclease activity, XPG contributes structurally to the formation of the NER complex (Constantinou et al., 1999; Evans et al., 1997a), and the structural presence of XPG – not its endonuclease activity – is necessary to permit ERCC1-XPF to cleave (Constantinou et al., 1999; Mu et al., 1997; Wakasugi et al., 1997). Recently, the crucial structure for this stimulatory activity was mapped to reside in the so-called spacer region of XPG, a >600 aa domain between its conserved nuclease domains that bears no clear homology to known proteins (Dunand-Sauthier et al., 2005; Thorel et al., 2004). Deletions in this domain abolish stable interaction with TFIIH and hence abrogate both the proper functioning of XPG in NER and its stimulation of ERCC1-XPF incision (Dunand-Sauthier et al., 2005; Thorel et al., 2004). In addition, the spacer domain is of critical importance to the preference of XPG to cleave bubble structures (Dunand-Sauthier et al., 2005; Thorel et al., 2004). XPG also contains a PCNA-binding domain, which could function to attract PCNA to the repair complex (Gary et al., 1997; Warbrick, 1998). However, even though XPG readily relocalises to damage sites in XP mutant cells from groups XP-A and XP-F (chapter 6), PCNA does not (Green and Almouzni, 2003; Katsumi et al., 2001; Miura and Sasaki, 1996), indicating that this domain alone is not sufficient to recruit PCNA.

XPG participates in several other pathways besides NER. At least one of the functions of XPG outside NER is essential, as knockout XPG mice are not viable (Harada et al., 1999), whereas XP-G patients expressing truncated XPG can live (albeit with a much shortened lifespan). Indeed, no XP-G patients exist that are null for XPG. In XP-G patients, the small fragment of XPG expressed must be just enough to perform the essential function. It has been reported that the *S. cerevisiae* homolog of XPG, Rad2, is required for efficient RNA pol II transcription (Lee et al., 2002), but whether this is the critical role that causes the XPG-null lethality is not clear. XPG also plays a role in BER, where it activates the thymine glycol glycosylase NTH1 (Bessho, 1999; Klungland et al., 1999).

3.8 Order of binding of RPA, XPG and XPA

After the initial complex of XPC-hHR23B and TFIIH is formed, XPG, XPA and RPA are recruited to the NER complex (Riedl et al., 2003 and chapters 6 and 8). The order of their binding is unclear, and several reports present evidence for various different orders of binding of these respective proteins.

XPG first?

Interactions between TFIIH and XPG have been observed repeatedly (Araujo et al., 2001; Iyer et al., 1996; Mu et al., 1995). Of all NER factors XPG in fact displays the strongest interaction with TFIIH (Araujo et al., 2001) and XPG relocalises to local UV-induced damages in the absence of functional XPA and XPF (chapter 6). The suggestion from these data, i.e. that XPG is recruited to the NER complex predominantly by its interactions with TFIIH is corroborated by recent findings. As mentioned above, deletions in the spacer domain of XPG cause weakened interactions with TFIIH and a consequently reduced incorporation into the NER complex

(Dunand-Sauthier et al., 2005; Thorel et al., 2004). These observations provide support for the idea that XPG is the first factor to be recruited following TFIIH, before both XPA and RPA (de Laat et al., 1999). The obvious lack of a genetic RPA mutant has so far prevented *in vivo* experiments into NER complex formation in the absence of RPA, leaving the possibility open that the incorporation of RPA into the complex precedes, and maybe even is necessary for, that of XPG. The advent of siRNA knockdown techniques has now made it possible to create temporary mutants in otherwise essential genes; thus, assessing NER complex formation after knockdown of RPA should be able to provide insights into this matter.

RPA first?

How RPA is attracted to the NER complex is unclear. RPA interacts only weakly with TFIIH (Araujo et al., 2001) and has not been reported to interact with XPC, ruling out a clear-cut recruitment via protein-protein interactions in the early stages of complex formation. RPA does interact with XPG (He et al., 1995) but this observation merely creates a chicken-and-egg type of question, i.e. both proteins may assist in recruiting the other, once the first of them is in the complex – but which is the first? Also the findings by Tapias and co-workers from the group of Egly are not conclusive. When added to ternary complexes of damaged DNA, XPC and TFIIH only the simultaneous addition of RPA, XPA and XPG led to further opening of the helix (Tapias et al., 2004); the possibility that RPA binds first before being activated by the arrival of XPA and/or XPG is however not ruled out by these observations.

In severe XP-B/CS mutant cells, the XPB subunit of TFIIH is presumed to be inactive in NER helix unwinding (Hwang et al., 1996), resulting in incomplete opening of the helix. Relocalisation of RPA to locally induced UV damage in these cells is nevertheless efficient (M. Volker, unpublished observation) suggesting that full opening around the lesion is not required to allow incorporation of RPA into the NER complex. As mentioned above, RPA can position and stimulate incision by XPG when RPA is pre-bound to a DNA substrate (de Laat et al., 1998b), suggesting that RPA is required to be present in the NER complex before XPG arrives. It has recently been observed that incomplete helix opening in TTD-A cells precludes XPA from being incorporated into the NER complex *in vivo* (Coin et al., 2006); it will thus be interesting to observe the behaviour of RPA following local UV irradiation in TTD-A cells.

Taken together, most data seem to indicate that the small stretches of ssDNA created by XPC-hHR23B and (even not fully functional) TFIIH and/or its weak protein-protein interactions with TFIIH are sufficient for RPA to join the NER complex. This is supported by the observation that following local UV irradiation, RPA efficiently relocalises to the subnuclear UV-damaged regions even in the absence of XPA or XPG (chapter 8). Given these observations and given the role RPA has in governing the endonucleases, it is conceivable that RPA is the first protein following TFIIH to be recruited to the NER complex.

XPA first?

Based on *in vitro* experiments in which (i) XPA was necessary to allow binding of both RPA and XPG to the complex, and (ii) incorporation of RPA stimulated binding of XPG, Riedl and co-workers suggested the order XPA → RPA → XPG (Riedl et al., 2003). However, as mentioned above and shown in chapters 6 and 8, local UV irradiation experiments using XP cells show recruitment of XPG and RPA to the NER complex to be independent of XPA, indicating that XPA incorporation into the complex is at least no prerequisite for the recruitment of RPA and

XPG and might not even precede one or both proteins. Conversely, XPG is dispensable for the incorporation of XPA into the NER complex (chapter 6), although it is possible that a truncated fragment of XPG present in the XP-G/CS cells used in these experiments is responsible for the observed recruitment of XPA.

No fixed order of binding of XPG, RPA and XPA?
Maybe the most satisfactory explanation for the inconsistent findings in the order of binding of XPG, RPA and XPA to the NER complex is that these factors bind in no specific order, and the idea of a fixed and strictly sequential order of binding in this phase of complex formation is incorrect. Instead, binding of any one of these factors to the complex could facilitate binding of the others by enforcing certain conformational changes (Riedl et al., 2003; Tapias et al., 2004) as well as through protein-protein interactions. The abovementioned findings from the group of Naegeli also indicate that XPA and RPA fulfil complementary roles in damage verification, without a specific requirement for either XPA or RPA to be present in the complex before the other protein (Missura et al., 2001).

In a recent publication, Politi and co-workers show that in a simple model 'disordered' binding of two factors at the second or third step of NER results in somewhat faster repair than strict sequential assembly (Politi et al., 2005). Although the authors do not describe models in which more than two factors bind in such way, it is conceivable that a similar disordered binding of three factors during one step would result in a similarly enhanced repair rate and thus present an advantage to the cell.

3.9 ERCC1-XPF

The second NER endonuclease consists of ERCC1 (33 kDa) and XPF (103 kDa), which mutually stabilise each other to form a heterodimer. ERCC1-XPF has a structural specificity complementary to that of its counterpart XPG, making a nick in ssDNA-to-dsDNA transitions at the 5' end of the ssDNA near the dsDNA (de Laat et al., 1998a; Sijbers et al., 1996). In contrast to XPG which cuts close to the lesion at the 3' side, ERCC1-XPF cuts further away (15-24 nt) at the 5' side of the lesion (Matsunaga et al., 1995; Sijbers et al., 1996). Although the XPF subunit contains the active nuclease domain (Enzlin and Scharer, 2002), the presence of ERCC1 is indispensable for nuclease activity. Additionally, the middle domain of ERCC1 interacts with XPA (Bessho et al., 1997; Li et al., 1995) and it was recently proposed that ERCC1 is also responsible for the interactions with DNA (Tripsianes et al., 2005).

Incorporation of ERCC1-XPF into the NER complex, allowing it to perform incision, depends on functional XPA (Li et al., 1995 and chapter 6) and the physical presence but not the nuclease activity of XPG (Constantinou et al., 1999; Mu et al., 1997; Wakasugi et al., 1997). While according to some reports, XPG in contrast is still capable of making an incision in the absence of ERCC1-XPF, giving rise to an uncoupled 3' incision (Evans et al., 1997b; Mu et al., 1996), a requirement for nuclease-active ERCC1-XPF in order for XPG to be able to incise has also been reported (Tapias et al., 2004). It has been shown that in the case of dual incision the cut by XPG is made prior to the cut by ERCC1-XPF (Evans et al., 1997b; Mu et al., 1996). All *in vitro* and *in vivo* evidence indicates that ERCC1-XPF is the final factor to be included in the NER complex before dual incisions are made.

Although uncommon, XP-F patients do exist; in contrast until recently no patients were known carrying mutations in ERCC1. When the first ERCC1 patient was reported he showed a dramatic phenotype lacking growth and development and died at the age of 13 months (N.G.J. Jaspers, manuscript in preparation). These observations correlate well with the phenotype of ERCC1-/- mice, which show a more severe phenotype (including neonatal lethality) than other NER-deficient mice (Friedberg and Meira, 2006; McWhir et al., 1993; Weeda et al., 1997) indicating that ERCC1 and XPF have one or more important functions outside NER.

ERCC1-XPF is known to be involved in several other repair pathways apart from NER, but the details of these roles remains to be elucidated. Relatively well-characterised is the involvement of ERCC1-XPF in the repair of interstrand crosslinks (ICL), for which it is essential (Busch et al., 1997; Kuraoka et al., 2000; Niedernhofer et al., 2004). Additionally, ERCC1-XPF is implicated in homologous recombination. In *S. cerevisiae*, mutant *rad10* or *rad1* (the homologs of ERCC1 and XPF, respectively) interferes with mitotic recombination (Aguilera and Klein, 1989; Klein, 1988; Prado and Aguilera, 1995; Schiestl and Prakash, 1988; Schiestl and Prakash, 1990; Zehfus et al., 1990). Since the effect of *rad10-rad1* double mutants is not more severe than that of single mutants it is very likely that also in this process ERCC1-XPF acts as a dimer, i.e. a structure-specific endonuclease. Also in mammalian cells, ERCC1 mutations result in a defect in the resolution of intermediates of homologous recombination (Sargent et al., 2000; Sargent et al., 1997). Furthermore, ERCC1-XPF is required for targeted gene replacement in mouse embryonic stem cells (Niedernhofer et al., 2001), and it is involved in telomere maintenance, removing the 3' overhang from uncapped telomeres (Zhu et al., 2003).

The combination of an inability to deal with intermediates in ICL repair and in HR during mitosis and defective telomere maintenance is likely to explain why ERCC1 and XPF null mice and patients display a much more severe phenotype than 'pure NER' knockouts or XP patients.

3.10 DNA resynthesis and ligation

After dual incision has taken place and the damaged oligo is removed, DNA resynthesis can commence, followed by ligation. In *in vitro* repair reactions the proteins involved in DNA replication during S-phase are capable of adequately carrying out these steps (Aboussekhra et al., 1995; Araujo et al., 2000; Shivji et al., 1995); therefore, it is generally assumed that they occur in a way and order similar to DNA replication.

As an early step PCNA is recruited, presumably shortly after or together with RF-C, because RF-C is the protein that loads PCNA onto DNA. This ATP-dependent loading occurs specifically at ds- to ssDNA transitions with a 3'-OH end, a structure characteristically formed after 5' incision by ERCC1-XPF and oligomer removal. In XP cells that cannot execute dual incision, PCNA does not associate with chromatin – i.e., does not participate in NER – after UV (Aboussekhra and Wood, 1995; Miura et al., 1992; Miura et al., 1996), an observation that is consistent with the idea that primed DNA resulting from dual incision is essential for PCNA to bind to DNA. The homotrimeric ring-shaped PCNA encircles DNA and acts as a clamp that slides along DNA. PCNA interacts with the replicative DNA polymerases δ and ε (reviewed in Maga and Hubscher, 2003) and enhances their processivity during replicative DNA synthesis (Maga and Hubscher, 1995; Podust and Hubscher, 1993; Prelich et al., 1987) by keeping them tethered to the DNA.

In *in vitro* NER systems, each of the DNA polymerases δ and ε can resynthesise the excised strand (Aboussekhra et al., 1995; Araujo et al., 2000; Shivji et al., 1995). Little research has been done as yet to determine whether there is discrimination between the two polymerases and if so, what the basis is for such discrimination. Both these polymerases have a high fidelity, making NER DNA resynthesis essentially error-free. Finally, *in vitro* experiments have shown that the remaining nick can be sealed by DNA ligase I (Aboussekhra et al., 1995; Araujo et al., 2000), which is also involved in ligation of the lagging-strand Okazaki fragments during normal cellular DNA replication. In chapter 10, *in vivo* research is presented addressing the involvement of DNA pol δ and pol ε and DNA ligase I in NER.

Chapter 4

More interplay between NER and transcription: inhibition of transcription following DNA damage

4 More interplay between NER and transcription: inhibition of transcription following DNA damage

Although NER and transcription are quite distinct processes several intimate links exist between them, two of which – TCR and the dual role of TFIIH in NER and transcription – have been described in chapters 2 and 3. A third link between NER and transcription is the temporary inhibition of transcription that occurs after the introduction of several types of DNA damage.

4.1 Transcription inhibition *in cis* and *in trans*

Two fundamentally different mechanisms can underlie an inhibition of transcription. First, transcribing RNA pol II may stall on a lesion (the same phenomenon that is thought to initiate TCR). This direct inhibition is referred to as *cis*-inhibition and is known to occur for various types of lesions, e.g. UV-induced photolesions (Giorno and Sauerbier, 1976). It is tempting to envisage TCR coming about as a direct consequence of this stalling, i.e. *cis*-inhibition of transcription. However, the severity of transcription-interference by a lesion does not correlate with its rate of removal by TCR (McGregor et al., 1995) indicating that other factors – subsequent steps in the TCR pathway, such as the ease of removal of RNA pol II from the lesion, or damage verification – are also important in determining its repair kinetics.

In addition to inhibition of transcription *in cis*, several observations indicate the existence of another mechanism, resulting in transcription inhibition *in trans*. In normal cells transcription returns to its normal levels within several hours following the induction of DNA damage (a phenomenon usually referred to as recovery of RNA synthesis or RRS). Remarkably even XP-D cells lacking TCR of UV lesions (van Hoffen et al., 1999) display complete RRS 24 hours after UV, albeit a low dose (van Hoffen et al., 1999). In contrast, in cells from CS and XP/CS patients transcription remains permanently inhibited following UV irradiation (Mayne and Lehmann, 1982; Moriwaki et al., 1996; Rockx et al., 2000; van Hoffen et al., 1999) or NA-AAF treatment – despite the complete repair of the NA-AAF-induced dG-C8-AF adducts by GGR (van Oosterwijk et al., 1996a; van Oosterwijk et al., 1996b). It is thought that this phenomenon contributes considerably to the high cell killing by UV irradiation in these cells (Andrews et al., 1978; van Hoffen et al., 1999), and underlies the sensitivity of CS cells to NA-AAF (van Oosterwijk et al., 1996b).

Other experiments show that the *trans*-effect actually occurs through inhibition of transcription at the initiation level. Using cell extracts competent for transcription initiation on plasmids Rockx and co-workers showed that in cell extracts from UV-irradiated cells, transcription did not initiate from plasmids that were themselves not damaged (Rockx et al., 2000). *In sum*, there is a number of indications arguing for additional inhibition of transcription acting *in trans*; more specifically, acting at the initiation level.

Finally, this phenomenon appears to be exclusively linked to lesions that are (at least potentially) subject to TCR, as UV-induced photolesions (Mellon et al., 1987) as well as dG-C8-AF adducts (van Oosterwijk et al., 1996a) are a target for TCR, although in the case of dG-C8-AF, repair by TCR is masked in normal cells due to the higher kinetics of repair via GGR (van Oosterwijk

et al., 1996b) whilst these lesions at the same time induce an overall reduction in transcription (Mayne and Lehmann, 1982; van Oosterwijk et al., 1996a; van Oosterwijk et al., 1996b). On the other hand, publications reporting TCR of oxidative lesions such as thymine glycols and 8-oxoguanine have been retracted or are under strong suspicion, and the introduction of such lesions does not lead to a reduction in overall transcription initiation (D.A.P. Rockx, unpublished observations).

4.2 A role for TFIIH in transcription inhibition *in trans*?

The dual involvement of TFIIH in transcription and NER lies at the basis of the so-called 'TFIIH-shuttling' model (van Oosterwijk et al., 1996b). In this model, in the presence of DNA damage TFIIH is preferentially tethered to repair sites, depleting it from sites of transcription initiation and so resulting in reduced transcription. *In vitro* research provided conflicting evidence on this matter. On the one hand, in a HeLa cell-free assay optimised to support NER and transcription simultaneously, Satoh and Hanawalt found that NER and transcription did not interfere with each other (Satoh and Hanawalt, 1996). In contrast, in yeast cell extracts capable of both NER and transcription You and co-workers found transcription to be inhibited when the system was simultaneously presented with NER substrates (You et al., 1998). Addition of TFIIH relieved this inhibition, leaving the authors to conclude that the preferential mobilisation of TFIIH to sites of repair was the primary cause of the transcriptional inhibition (You et al., 1998).

Subsequent experiments however have provided more evidence against the TFIIH-shuttling hypothesis. First, Rockx and co-workers tested the hypothesis in the abovementioned *in vitro* transcription assay (Rockx et al., 2000). The prior inclusion of additional TFIIH was found to have a non-specific positive effect on transcription initiation in extracts from both unirradiated and irradiated cells (Rockx et al., 2000). Additionally, using GFP-tagged XPB, Hoogstraten et al. found that the maximum fraction of TFIIH that could be tethered to repair was ~40% (Hoogstraten et al., 2002). Since in non-damaged cells only 20-40% of TFIIH is involved in transcription at any one time (Hoogstraten et al., 2002) these results together reinforce the idea that recruitment of TFIIH to repair sites does not cause an inhibition of transcription. Finally, we tested whether TFIIH, or any other freely diffusible factor, is involved in propagating the signal for transcription inhibition throughout the nucleus. One early experiment suggested that transcription inhibition not only takes place *in trans*, but also spreads throughout the nucleus. As early as 1967, Takeda and co-workers used a UV microbeam to induce UV damage in a small volume of HeLa cell nuclei and measured the effect of this irradiation on transcription with 3H-uridine pulse-labelling. Transcription decreased in the entire nucleus, suggesting that transcription was inhibited *in trans* and the signal for inhibition was distributed by a factor that can freely diffuse through the nucleus (Takeda et al., 1967). However, using a different UV irradiation method and run-off transcription labelling with BrUTP, followed by immunofluorescent labelling, we were unable to reproduce a nucleus-wide transcription inhibition. Instead, we found the inhibition to be confined to the area of damaged DNA, and we concluded that the *trans*-inhibition in fact has a limited range (chapter 7).

4.3 What causes the transcription inhibition *in trans*?

As yet the exact cause for the *trans*-effect of transcription inhibition is not known, but some indications exist. Most notably, following UV irradiation, the hypophosphorylated initiating form of RNA pol II (RNA pol IIa) is reduced and the amount of hyperphosphorylated elongating form (RNA pol IIo) increases (Rockx et al., 2000), coinciding with the reduction in transcription. Conversely, as transcription returns to its pre-UV levels, RNA pol IIa reappears (Rockx et al., 2000). In sharp contrast, in CS-A and -B as well as XP-A cells both transcription and the levels of RNA pol IIa remain low after UV (Rockx et al., 2000). Thus, the *trans*-effect of transcription inhibition reveals itself as a shift in RNA pol II from predominantly the initiating to the non-initiating/elongating form. Whether the stalling of RNA pol II on a lesion is the leading cause for this shift in phosphorylation state or whether the shift is brought about by other means, e.g. a UV-sensing signalling pathway, is unknown. It is however curious that only those DNA lesions that are a target for TCR appear to give rise to *trans*-inhibition of transcription (see above); it is therefore tempting to speculate that it is the stalling of RNA pol II that, directly or indirectly, triggers the transcription inhibition. The conceptual problem that arises from models in which another damage sensor (such as a signalling pathway utilising receptors in the cell membrane) evokes the *trans*-effect is that this sensor seemingly is able to predict if a type of DNA damage is a target for TCR.

Chapter 5

Chromatin, chromatin remodelling and NER

5 Chromatin, chromatin remodelling and NER

It has long been recognised that the major obstacle to overcome for a repair system – indeed, for any process intimately interacting with DNA – in a human nucleus is the chromatin structure into which the DNA is condensed. At the lower level of chromatin organisation, 146 bp of DNA are wrapped around histone octamers (two each of the histones H2A, H2B, H3 and H4) to form the nucleosome core; these in turn are further compacted into higher-order structures such as the 10-nm fibre and the 30-nm fibre. These higher levels of chromatin structure are still poorly characterised, and their effects on repair have yet to be investigated.

The effect of the lower levels of chromatin structure on repair has been best characterised for the NER pathway, both *in vivo* and *in vitro*. *In vivo*, chromatin interacts with NER at two levels. At the level of the nucleosomes DNA lesions can be situated in linker DNA or in the histone core, whereas at the level of higher-order structure DNA lesions might reside in (transcriptionally) active or inactive DNA. A large number of studies conducted in the last decades have consistently found that lesions in the linker of nucleosomes and in active DNA are repaired significantly faster. This is not surprising when one realises that these DNA structures are more open and therefore the repair system has better access to detect and repair them. In the case of TCR, apart from better access to the lesion due to more relaxed chromatin structures, NER also enhances its repair rates actively, employing RNA polymerase stalled on a lesion as a damage sensor (see chapter 2.1).

From the early 90s of the previous century onward, *in vitro* systems have been available to complement *in vivo* research. Utilising cell-free extracts, reconstituted systems with purified proteins or a combination of both, repair from naked DNA has been compared with repair from chromatinised templates. Since the level of DNA packaging in these experiments does not exceed that of the nucleosome, these assays measure exclusively the difference in repair rates between linker and core DNA. As *in vivo*, repair is more rapid from naked DNA than from chromatinised templates (Araki et al., 2000; Hara et al., 2000; Sugasawa et al., 1993; Wang et al., 1991).

For a long while it has been known that during NER *in vivo*, chromatin rearrangements are made (Smerdon and Lieberman, 1978). It was postulated that these rearrangements were at least partly aimed at opening inaccessible chromatin domains to repair; similarly, following repair these rearrangements could restore the chromatin structure to its original state (Smerdon and Lieberman, 1978). The model that was derived from these and corresponding observations was first postulated by Smerdon as the access-repair-restore (ARR) model and has proven very useful for the understanding and study of NER in the context of chromatin (Smerdon, 1991).

Although originally chromatin was thought of as a rigid structure with the sole purpose of compacting DNA, it has become clear that in fact, chromatin is a highly dynamic, 'fluid' structure that actively influences DNA metabolising processes such as transcription. One major factor contributing to the accessibility of chromatin is a group of enzymes referred to as chromatin remodellers, whose function is to change the structure of chromatin in order to alleviate its restrictive effects. Chromatin remodelling enzymes are divided into two major classes: ATP-dependent non-covalent remodellers and enzymes that covalently link attachments to histone tails. Following the investigations of the effects of the chromatin structure on NER, the effects of its remodelling have likewise been assayed. The effect that several chromatin remodelling enzymes have on NER, both *in vitro* and *in vivo*, is discussed below.

5.1 ATP-dependent non-covalent chromatin remodelling

The type of chromatin remodelling performed by the ATP-dependent non-covalent remodellers involves the breaking and reformation of contacts between histones and DNA, resulting in a mobilisation of nucleosomes along the chromatin template. All non-covalent chromatin remodellers belong to the SWI2/SNF2 superfamily of ATPases. Based on the identity of their catalytic ATPase subunit 3 subfamilies can be discerned: SWI/SNF, ISWI, and Mi-2. The prevailing mechanistic model for these enzymes is that they interconvert chromatin between different states in a random manner. As such, the outcome of conversion may be beneficial or detrimental to processes acting on the chromatin. Furthermore, contacts between DNA and histones will only be disturbed transiently, meaning that any positive (or negative) effect on the accessibility of the DNA can easily get lost. It has for instance been found that for transcription the effects of SWI/SNF remodelling are temporal, unless the opened structure is bound by a transcription factor (Owen-Hughes et al., 1996).

SWI/SNF and ISWI

So far, two ATP-dependent chromatin remodelling enzymes have been found to stimulate NER *in vitro*: SWI/SNF (Hara and Sancar, 2002; Hara and Sancar, 2003) and ACF (Ura et al., 2001), which consists of ISWI and acf-1. Both factors enhance transcription by 'loosening up' the chromatin structure. In addition, evidence has been presented that nucleosomal inhibition of photoreactivation by *E. coli* photolyase (Gaillard et al., 2003), and incision by T4 endonuclease V and *Micrococcus luteus* UV endonuclease (Lee et al., 2004) is relieved upon the addition of ISWI (Gaillard et al., 2003) and SWI/SNF (Gaillard et al., 2003; Lee et al., 2004). These data strongly suggest that the stimulation by chromatin remodellers on human NER is not specific for human repair or for the NER system.

CSB

A special case worth noting is the CSB protein, of which the ATP-dependent chromatin remodelling capacity appears not to be strictly required for functional TCR (Citterio et al., 2000); discussed in chapter 2.1. This may suggest that instead, CSB activity is required for another process; indeed, several observations indicate a role for CSB in transcription, where it might stimulate RNA pol II to proceed past transcriptional pause sites and/or other obstructions (Balajee et al., 1997; Lee et al., 2002; van Gool et al., 1997).

5.2 Histone tail modifications

The second type of chromatin remodellers encompasses enzymes that covalently link attachments to histone tails. These modifications include acetylation, methylation, ubiquitination and SUMOylation, and others. The different modifications are thought to form a 'histone code' which fine-tunes and orchestrates the binding of transcription factors and chromatin-remodelling enzymes, resulting in either activation or repression of transcription.

It would lead to far to discuss in detail the various effects that each of these modifications can have. In brief, ubiquitination or SUMOylation of histones is in general associated with an increase or decrease in transcriptional activity, respectively (as is the case for transcription

factors). Methylation appears to be an irreversible modification that is primarily used to maintain a certain repressed chromatin state for prolonged periods of time, such as in terminally differentiated cells (the permanent repression of *DDB2* in cultured rodent cells mentioned in chapter 3.1 being an example). In contrast, acetylation is a reversible dynamic process that decreases interactions between nucleosomes and between the tails and linker DNA, thus leading to an increased accessibility to the DNA. For example, it has long been known that acetylation of histones generally results in increased transcription. Constitutive acetylation of histones H3 and H4 is also associated with increased rates of NER and DSBR. Correspondingly, the opposite process – deacetylation – is associated with repression of transcription. Although the effect that chromatin remodelling has on transcription has been studied for a long time, its effect on repair is only beginning to emerge.

Histone acetylation and NER

Of the various covalent modifications of the histone tails, only acetylation activity has so far been associated with NER. In the first hours following UV irradiation and in the presence of sodium butyrate, histones are hyperacetylated in human fibroblasts (Ramanathan and Smerdon, 1986) resulting in higher rates of NER (Ramanathan and Smerdon, 1986). In HeLa cells histones H3 and H4 are acetylated following UV treatment even in the absence of sodium butyrate (Brand et al., 2001), although in this situation the effect on NER was not tested. These data suggest that UV-induced (hyper)acetylation of histones is a cellular response to facilitate repair. *In vitro* there is as yet only circumstantial evidence to corroborate this: excision of a cisplatin adduct by Chinese hamster ovary cell-free extracts was found to be 2-fold more efficient when the DNA was wrapped in purified human histones compared to recombinant human histones (Wang et al., 2003). The human histones in this situation may have been acetylated, facilitating repair, whereas the recombinant histones were most certainly not.

Interestingly, an intimate link appears to exist between histone acetylation and NER damage recognition. Firstly, the histone acetyltransferase (HAT) p300/CBP interacts with both DDB1 (Rapic-Otrin et al., 2002) and DDB2 (Datta et al., 2001). Secondly, two human HAT-containing complexes display an affinity for UV-damaged DNA: TFTC (Brand et al., 2001) and STAGA (Martinez et al., 2001). Both these complexes contain the spliceosome-associated protein SAP130, which shares a high homology (>50% similarity) with the DDB1 subunit of UV-DDB. SAP130 interacts with DDB1, and the STAGA complex interacts with both subunits of UV-DDB. Both TFTC and STAGA bind preferentially to UV-damaged DNA, in the case of TFTC causing acetylation of histone H3 assembled on the damaged DNA. Exactly through which protein(s) binding to damaged DNA occurs is not clear yet. Brand and co-workers found that SAP130 as a separate factor can bind to UV-damaged DNA, and this binding activity is stimulated in the TFTC complex (Brand et al., 2001). On the other hand, Martinez and co-workers reported that SAP130 in the absence of STAGA compounds shows hardly any preference for UV-damaged DNA and accordingly, STAGA without SAP130 still displays an affinity for UV-damaged DNA (Martinez et al., 2001).

Despite these (as yet) contradictory findings it is clear that there might be a strong interplay between the 'access' and 'repair' steps as described by the ARR model, with chromatin modifying enzymes displaying an affinity for damaged DNA and damaged DNA-recognising proteins interacting directly with histone acetylases.

5.3 Non-chromatin remodellers assisting NER in vivo

Gadd45

The *GADD* genes form a group of genes that are specifically induced after cells are growth-arrested or exposed to DNA damaging agents (Fornace et al., 1989). The product of the *GADD45* gene, Gadd45, has been linked to NER via several observations. The Gadd45 protein displays a specific affinity for UV-damaged and hyperacetylated nucleosomal DNA and can modulate the accessibility of such DNA-nucleosome complexes (Carrier et al., 1999). Human tumour cells in which the Gadd45 levels were knocked down by Gadd45 antisense expression showed reduced repair of UV-damaged DNA (Smith et al., 1996). Gadd45-/- MEFs have reduced UDS following exposure to UV, coinciding with a reduced rate of CPD and 6-4PP removal (Smith et al., 2000). Finally, Gadd45-/- murine keratinocytes show a strong defect in the repair of CPD compared to wild type murine keratinocytes after irradiation with low doses of UVB (Maeda et al., 2002). Interestingly, p53 regulates *GADD45* (reviewed in Zhan, 2005) which might lead to responses of p53-/- cells to DNA damage being erroneously attributed to a direct effect of p53-deficiency (see also below).

HMGN1

HMGN (high-mobility group N) proteins are not classical chromatin-remodelling factors, but are capable of destabilising higher-order chromatin structures by targeting two main elements known to compact chromatin, histone H1 and the N-terminal tail of histone H3 (reviewed in Bustin, 1999; Bustin, 2001). In doing so, they increase the rate of transcription and replication (Crippa et al., 1993; Trieschmann et al., 1995; Vestner et al., 1998). HMGN1-/- mice show decreased rates of CPD removal from the transcribed strand of active genes (Birger et al., 2003). Whether this effect is correlated to overall lower transcription rates in the absence of HMGN1 and hence, lower TCR rates, or whether the more relaxed chromatin in HMGN1+/+ cells allows the TCR machinery to be more efficiently targeted to RNA pol II stalled on lesions is not known.

p53

p53 has been known for a long time to play an important role in NER, most notably by regulating the expression levels of DDB2 and XPC (discussed in chapter 3.4). More directly, p53 has been reported to associate with TFIIH modulating its activity in transcription and NER (Leveillard et al., 1996; Wang et al., 1995), although there has been no follow-up to these initial observations.

Surprisingly however, recently p53 has been implicated in NER as a global chromatin accessibility factor (Allison and Milner, 2004; Rubbi and Milner, 2003). Rubbi and Milner reported that chromatin relaxation following UV occurs in normal, XP-A, -C or -E but not in p53-null human fibroblasts, because p53-deficient cells were specifically defective in acetylation of histone H3 after UV irradiation (Rubbi and Milner, 2003). The authors also reported that this relaxation of chromatin occurs in the entire nucleus even if only part is UV-irradiated (Rubbi and Milner, 2003). Furthermore, they found p53 and p300 to colocalise with sites of NER using immunofluorescent labelling of p53 or p300 and detection of transient ssDNA to visualise patches of NER, followed by image analysis (Rubbi and Milner, 2001; Rubbi and Milner, 2003). However, using more conventional methods such as subnuclear UV irradiation followed by indirect immunofluorescent labelling of p53, p300 and CPD and 6-4PP, no such colocalisation has been observed (Fitch et al., 2003a; M. Volker, M. Vrouwe, unpublished observations).

Furthermore, since Gadd45 is under control of p53 (reviewed in Zhan, 2005) the cells used by Rubbi and Milner (2003) could also be deficient in a Gadd45-dependent process that only indirectly depends on p53.

5.4 Post-repair chromatin restoration

CAF-1 and Asf1

In contrast to the pre-repair ('access') chromatin remodelling, post-repair ('restore') chromatin remodelling has not been extensively studied. The best-studied factor in the restoration of the chromatin structure is CAF-1, a histone chaperone that is involved in depositing nucleosomes on newly synthesised DNA during replication (reviewed in Mello and Almouzni, 2001). Additionally, *in vitro* CAF-1 is capable of specifically assembling nucleosomes onto damaged DNA that has been repaired by NER (Gaillard et al., 1996), and CAF-1 is recruited to UV-damaged DNA both *in vitro* (Moggs et al., 2000) and *in vivo* (Green and Almouzni, 2003). This recruitment depends on functional NER (Green and Almouzni, 2003) and crucially depends on PCNA (Green and Almouzni, 2003) which is involved in the resynthesis stage of NER (chapter 3.10). It therefore seems plausible that CAF-1 is responsible for the local restoration of the chromatin structure directly following repair. Its association with PCNA could imply that CAF-1 is also involved in the restoration of chromatin following other repair pathways that utilise PCNA in their DNA resynthesis stages, such as long patch BER or homologous recombination.

Recently it was found that *in vitro*, the histone chaperone Asf1 can synergistically facilitate the nucleosome assembly by CAF-1 during NER (Mello et al., 2002). In contrast to CAF-1, Asf1 does not associate specifically with damaged DNA nor is it recruited to damaged DNA during repair (Mello et al., 2002). It is therefore thought that Asf1 acts upstream of CAF-1 by supplying CAF-1 with histones so that CAF-1 can efficiently execute its function (Mello et al., 2002).

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Chapter 6

Sequential assembly of the nucleotide excision repair factors in vivo

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6 Sequential assembly of the nucleotide excision repair factors in vivo

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Summary

Here, we describe the assembly of the nucleotide excision repair (NER) complex in normal and repair-deficient (xeroderma pigmentosum) human cells, employing a novel technique of local UV irradiation combined with fluorescent antibody labeling. The damage recognition complex XPC-hHR23B appears to be essential for the recruitment of all subsequent NER factors in the preincision complex, including transcription repair factor TFIIH. XPA associates relatively late, is required for anchoring of ERCC1-XPF, and may be essential for activation of the endonuclease activity of XPG. These findings identify XPC as the earliest known NER factor in the reaction mechanism, give insight into the order of subsequent NER components, provide evidence for a dual role of XPA, and support a concept of sequential assembly of repair proteins at the site of the damage rather than a preassembled repairosome.

Introduction

In eukaryotes, nucleotide excision repair (NER) is a versatile and highly conserved repair system capable of removing a wide range of DNA lesions that distort the stacking of the DNA double helix, including the shortwave ultraviolet (UV) light-induced cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP). In humans, repair of UV-induced photolesions is entirely dependent on NER, and mutations in NER proteins have been associated with the inherited disorder xeroderma pigmentosum (XP) (Bootsma et al., 1998). XP patients suffer from severe photosensitivity and a high incidence of sunlight-induced skin cancers. NER consists of a general pathway termed global genome repair (GGR) that removes lesions from the entire genome and a specialized pathway referred to as transcription-coupled repair (TCR). Repair by TCR is confined to DNA lesions in the transcribed strand of transcriptionally active genes and strictly depends on ongoing transcription by RNA polymerase II (RNAPolII) (Leadon and Lawrence, 1991; Venema et al., 1992). Examination of the repair kinetics of structurally different DNA lesions has disclosed that TCR functions as an efficient repair system for transcription-blocking lesions that are poorly repaired by GGR (Hanawalt, 1995). In the case of UV-induced photolesions, repair of 6-4PP is fast throughout the genome and is dominated by GGR. In contrast, GGR of CPD is relatively slow, but TCR causes accelerated removal of this lesion from the transcribed strand of expressed genes (van Hoffen et al., 1995).

Cell fusion experiments have revealed seven XP genetic complementation groups (XP-A through XP-G) that represent different proteins in the NER pathway. Among the various complementation groups, XP-C is unique, as only GGR is compromised in this group (Venema et al., 1991). The photosensitive inherited disorder Cockayne syndrome (CS), on the other hand, is associated with defective TCR, while GGR is unaffected (van Hoffen et al., 1993; Venema et al., 1990). At the cellular level, the two CS genetic complementation groups (CS-A and CS-B) are characterized by a lack of recovery of inhibited RNA synthesis following exposure to DNA damaging agents, a phenomenon that has been related to defective TCR (Mayne and Lehmann, 1982; Venema et al., 1990).

Incision of damaged DNA is a multistep process involving recognition of the DNA damage followed by opening up of the DNA helix around the lesion, dual incision, and subsequent excision of the oligonucleotide containing the DNA lesion (de Laat et al., 1999). From in vitro biochemistry, it is not clear in which order various NER factors act in the reaction mechanism, particularly with respect to the first stages including the crucial damage recognition step. In addition, it is not evident what the organization of repair is in vivo – are NER factors preassembled in a NER holocomplex, in distinct subassemblies, or as individual factors that transiently interact at the site of the lesion? The identity of the damage recognition factor has been a matter of debate. Several putative candidates have been proposed, including the XPA-replication protein A (RPA) complex (Asahina et al., 1994; Li et al., 1995b), the XPC-hHR23B complex (Reardon et al., 1996, Batty and Wood, 2000; Yokoi et al., 2000), and the p48-p127 complex, also termed damaged DNA binding (DDB) protein (Chu and Chang, 1988; Tang et al., 2000). DDB has not been implicated so far in the damage recognition step in in vitro experiments. Results obtained in in vitro experiments are contradictory as to whether XPC-hHR23B or XPA-RPA is the principal damage recognition protein. Findings by Sugasawa and coworkers using *N*-acetoxy-2-acetyl-aminofluorene-adducted plasmids in an in vitro damage recognition-competition assay indicate that XPC-hHR23B is the earliest damage detector to initiate NER in vitro (Sugasawa et al., 1998). Furthermore, XPChHR23B specifically binds a small bubble structure with or without damaged bases, which is evidence that damage recognition for NER is accomplished through at least two steps. XPC-hHR23B first binds to a site displaying DNA helix distortion. Subsequently, the presence of injured bases is verified prior to dual incision (Sugasawa et al., 2001). In contrast, using DNA binding and repair assays on a single 6-4PP, Wakasugi and Sancar (1999) report that RPA and XPA are the initial damage-sensing factors of human excision nuclease. Assembly of the NER complex involves the recruitment of the basal transcription factor IIH (TFIIH) and the structure-specific endonucleases ERCC1-XPF and XPG (de Laat et al., 1999). TFIIH exerts a dual function in the cell, being an essential factor in RNAPolII transcription initiation and in NER (Drapkin et al., 1994; Feaver et al., 1993; Schaeffer et al., 1993). The core TFIIH complex consists of six polypeptides that are indispensable for both NER and transcription initiation. Two components of core TFIIH, i.e., the XPB and XPD proteins, are helicases responsible for unwinding the DNA helix in NER; the single-stranded DNA generated as an intermediate in the NER reaction is stabilized by RPA (de Laat et al., 1998). The identity of the proteins responsible for the binding of TFIIH to the site of DNA damage and their sequence of action have not been fully clarified yet. Recent findings by Yokoi et al. (2000) suggest that TFIIH interacts with XPC-hHR23B bound to damaged DNA, indicating that XPC-hHR23B attracts TFIIH to the lesion. Interestingly, association of TFIIH with DNA was observed in both wild-type and XP-A cell extracts but not in XP-C cell extracts, and XPC-hHR23B could restore the association of TFIIH with DNA in XP-C cell extracts. Whether this process requires functional XPA protein in vivo is not known.

Like most repair systems, NER faces the requirement to reach DNA lesions in any location of the genome independent of chromatin conformation or stage of the cell cycle. Hence, one can anticipate that repair enzymes function in a highly organized and dynamic fashion in the nuclear context, possibly as a preassembled complex, i.e., a nucleotide excision repairosome. Experiments aimed to demonstrate the occurrence of a preassembled repairosome in the yeast *Saccharomyces cerevisiae* have led to contradictory results. Svejstrup and coworkers (Svejstrup et al., 1995) showed that TFIIH might exist in a complex with NER proteins, whereas results by other investigators (Guzder et al., 1996) provided no evidence for a preassembled complex but rather support a model involving four NER subcomplexes. In mammals, Houtsmuller et al. provided evidence against a stable holocomplex (1999). In the present study, we have investigated the assembly of the NER incision complex in UV-irradiated human cells, employing a recently developed technique (M.J. Moné et al., submitted) to inflict UV damage in restricted parts of the nucleus in combination with immunofluorescent labeling. We are able to dissect the entry of various NER proteins at sites of UV damage and show that XPChHR23B is the first protein to bind to (photo)lesions.

Furthermore, we present evidence that XPC-hHR23B is necessary to recruit TFIIH to lesions and that this recruitment of TFIIH does not require functional XPA protein. Formation of the incision complex by the association of ERCC1-XPF and XPG to the preincision complex shows differential participation of XPA: binding of XPG occurs in the absence of XPA, whereas XPA is indispensable for the association of ERCC1-XPF. A second function of XPA might be the activation of the incision activity of XPG. Our findings indicate that NER is mediated by sequential assembly of repair proteins at the site of the DNA lesion rather than by the action of a preassembled repairosome.

Results

Distribution of NER Proteins in Nonexposed Cells

The nuclear distribution pattern of proteins implicated in the damage recognition and incision steps of NER was assessed by immunofluorescent labeling using antibodies raised against XPA, XPC, the XPB subunit of TFIIH, ERCC1, and XPG. In growing cells, fluorescent images of various proteins consisted of numerous small foci dispersed throughout the nucleus (Figure 1A); additionally, ERCC1 exhibited larger and brighter foci throughout the nucleus, which were also present in living cells (Houtsmuller et al., 1999). The numerous small foci might be induced by clustering of ERCC1 molecules during fixation. In growing cells, TFIIH displayed a homogeneous distribution pattern with a large number of small foci dispersed throughout the whole nucleus as reported previously (Grande et al., 1997), whereas in confluent cells TFIIH is concentrated in a smaller number of distinct foci of larger size, and parts of the nucleus became apparently devoid of TFIIH (Figure 1A). This difference in distribution of TFIIH between growing and confluent cells (observed in all cell strains tested) most likely relates to the ~5-fold lower level of transcription in human fibroblasts at confluency (Enninga et al., 1985), and hence the TFIIH-rich foci in confluent cells might represent storage sites from which TFIIH can be recruited. Similar results were obtained with an antibody directed against the p62 component of TFIIH. The patterns of all other tested NER proteins (XPA, XPC, XPG, ERCC1) did not change when cells reached confluency.

UV Light Changes the Distribution Pattern of TFIIH

Exposure of confluent human cells (normal, XP-A, XP-C, CS-B) to UV light (2 or 10 J/m^2) did not alter the distribution of XPA, XPC, ERCC1, or XPG proteins (data not shown). In contrast, UV irradiation markedly affected both the distribution and the intensity of the TFIIH fluo-

rescence in normal human fibroblasts, resulting in a homogeneous distribution and enhanced intensity. In growing cells, such change in TFIIH fluorescence was not manifest, most likely because alterations are masked by the homogeneous distribution of TFIIH in nonirradiated cells (Figure 1A). Figure 1B shows the TFIIH fluorescence at 30 min after UV exposure. In confluent cells, the fluorescence intensity went up with increasing UV dose, but, irrespective of the dose, TFIIH fluorescence changed as fast as 15 min after UV exposure and returned to that of nonirradiated cells within 2 hr. This change in TFIIH fluorescence must be elicited by a cellular response to UV damage, since we did not observe an alteration of fluorescence when UV-irradiated cells were kept at 0°C. Quantification of the fluorescence showed that the level increased ~50% within 15 min after UV, remained elevated until 1 hr after UV irradiation, and shifted back to that of nonirradiated cells within 2 hr. Western blot analysis of whole-cell extracts revealed that enhanced fluorescence signal was not due to an elevated amount of XPB (Figure 1C) or p62 (data not shown).

The fluorescence signal of TFIIH also changed upon UV irradiation of XP25RO (XP-A) cells completely defective in NER (Tanaka et al., 1990) (Figures 1B and 2). In contrast to normal cells, the TFIIH fluorescence in XP-A cells did not return to its pre-UV pattern. This demonstrates that the UV-induced change in TFIIH is independent of functional XPA protein but that the restoration of TFIIH fluorescence to its pre-UV state depends on functional NER. Remarkably, XP21RO (XP-C) cells defective in GGR only exhibited hardly any alteration of TFIIH fluorescence upon UV exposure (Figures 1B and 2), suggesting that the observed alteration of TFIIH fluorescence in normal human cells following UV exposure requires the presence of functional XPC. On the other hand, CS1AN (CS-B) cells defective in TCR but proficient in GGR displayed UV-induced changes in TFIIH fluorescence highly similar to those observed in normal human cells (Figures 1B and 2), indicating that the time-dependent change in nuclear distribution of TFIIH does not require TCR.

These results imply that specific steps in NER involving XPC are responsible for the observed changes in TFIIH fluorescence. However, from these experiments it is hard to distinguish whether increased accessibility of TFIIH to antibody is a result of its recruitment from storage sites to DNA damage or merely that alterations in protein configuration at sites of DNA damage (affecting recognition by the antibody) underlie the observed changes in fluorescence.

Distribution of NER Proteins after Local UV Irradiation of the Nucleus

To assess whether UV irradiation provokes intranuclear translocation of repair proteins to the sites of DNA photolesions, we exposed small parts of the nucleus to UV radiation ('local' irradiation) using a methodology described in detail by Moné et al. (submitted). For this purpose, monolayer cultures of human fibroblasts were covered with isopore polycarbonate filters with pore diameters of either 3 or 8 μm prior to UV irradiation. These filters block UV light with wavelengths shorter than 300 nm, and, consequently, only at sites of pores will UV damage be induced. Immunostaining with antibodies against CPD and 6-4PP (Mori et al., 1991) confirmed that nuclei contained only UV damage in local spots (Figures 3A and 3B). A run-on DNA synthesis assay in permeabilized repair-proficient human cells (VH25), carried out 20 min after local UV irradiation in the presence of biotin-tagged dUTP and subsequent labeling with FITC-conjugated avidin (Jackson et al., 1994), showed that DNA repair synthesis was restricted to these spots of damage (Figure 3C). This finding was corroborated by the observation that 2 hr after exposure to 30 J/m^2 of UV, spots of 6-4PP were not detected anymore in normal cells, indicating virtually complete repair of these photolesions. In nondividing NER-deficient XP-A cells, on the other hand, spots of 6-4PP remained visible even after 24 hr following local UV exposure.

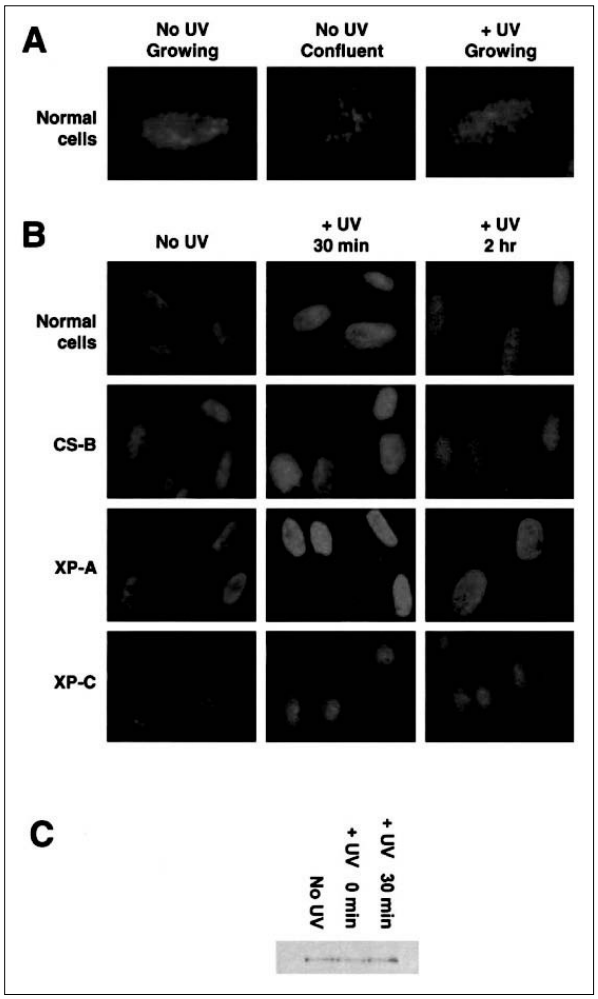
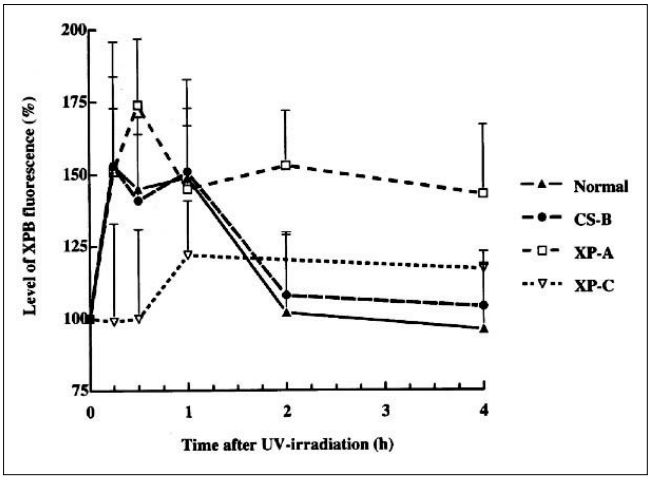


Figure 1. Altered Nuclear Distribution of TFIIH in Confluent Human Fibroblasts after Global UV Irradiation

(A) Growing or confluent normal human fibroblasts (VH25) were fixed and immunolabeled employing an antibody against XPB or immunolabeled at 30 min after UV irradiation (10 J/m^2). (B) Confluent normal human (VH25), CS-B (CS1AN), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were either fixed and immunolabeled using an antibody against XPB or exposed to UV (10 J/m^2) and immunolabeled 30 min or 2 hr later. (C) Western blot analysis of XPB. Confluent VH25 fibroblasts were mock or UV-treated, and protein extracts were prepared either immediately or 30 min after treatment. See the Appendix for a colour version of this figure.

Figure 2. Intensity of the TFIIH Immunofluorescent Signal in Confluent Cells as a Function of Time after UV Exposure

Normal human (VH25), CS-B (CS1AN), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were fixed at various times after global UV irradiation. The fluorescent signal of XPB in nuclei was quantified, normalized to the DAPI-stained nuclear area, averaged, and set to 100% at 0 hr. The error bars represent the SEM values of 30 nuclei.



Repair of photolesions in damage spots requires the assembly of the NER incision complex and hence the recruitment of TFIIH as well as other NER proteins. As shown in Figure 4, enhanced fluorescence signals of XPA, XPC, and TFIIH (XPB) emerged in spots of damage within 15 min following local irradiation of normal human cells (VH25), i.e., within the same time period as the change in fluorescent signal of TFIIH following global UV exposure. The NER proteins in the damage spots are most likely recruited from undamaged regions. This is indicated by comparison of the fluorescent signals in undamaged cells and locally UV-irradiated cells, revealing that the fluorescent signals of NER factors were clearly reduced in regions outside the damage spots (Figures 4 and 5A). These observations are consistent with the long retention times of GFP-tagged ERCC1 at sites of DNA damage determined in living cells (Houtsmuller et al., 1999). In addition to the aforementioned NER factors implicated in the preincision step, it is anticipated that the structure-specific endonucleases XPG and ERCC1-XPF involved in the 3′ and 5′ incision reaction, respectively, will be recruited to the site of damage. Indeed, XPG and ERCC1 proteins were enriched in damage spots when normal human fibroblasts were assayed 15 min after local UV exposure (Figure 6). A dose of 30 J/m^2 resulted in a profound reduction of XPG and ERCC1 throughout the nonirradiated part of the nucleus, and most of the fluorescence was confined to the damage spot.

Assembly of the Incision Complex

To assess whether NER occurs by sequential assembly of different repair factors at the site of DNA damage or by recruitment of a preformed repairosome, we examined the distribution of NER factors in various repair-deficient human cell lines after local UV irradiation. The first series of experiments aimed to clarify the roles of the putative damage recognition proteins XPA and XPC. Cells of an XP-A patient (XP25RO) completely deficient in repair of UV photolesions appeared to be fully capable of accumulating the XPC protein at spots of damage within 15 min after exposure. Concomitantly with this accumulation, a vast reduction of XPC in nonirradiated parts of the nucleus was observed, closely corresponding to what has been discerned in normal human cells (Figure 4). When the distribution of TFIIH (XPB) was assessed in locally UV-irradiated XP-A cells, it was evident that TFIIH too became enriched at damaged spots in the absence of a functional XPA protein, whereas the amount of TFIIH in nonirradiated parts of the nucleus was diminished. We considered the possibility that local UV damage may deplete TFIIH from the unirradiated areas in the nucleus. Even after high levels of local UV damage (six to eight spots of 30 J/m^2) and allowing XP-A cells to sequester TFIIH to DNA damage for up to 4 hr, part of TFIIH appeared to remain in undamaged areas of the nucleus (Figure 5B). This suggests that a subset of TFIIH is never sequestered by NER and persists in sufficient quantity to account for unaffected levels of transcription outside the damaged spots in locally UV-irradiated cells (M.J. Moné et al., submitted). Together, our results demonstrate that XPA is not required for the binding of TFIIH and XPC to a lesion.

In marked contrast to the findings in XP-A cells are the results achieved with XP-C cells. In XP21RO cells, neither the XPA protein nor TFIIH (Figure 4) was found to accumulate in spots of local damage at any time point after UV exposure. As a matter of fact, the nuclear distribution of both proteins was unaffected by the UV irradiation, suggesting that functional XPC protein is essential for the formation of the incision complex.

Next, we addressed the question of whether the accumulation of the incision endonucleases XPG and ERCC1-XPF is dependent on the presence of functional XPA and XPC factors. In locally UV-irradiated XP-A cells, a strong accumulation of XPG in damage spots coincided with a strong reduction of the protein in nonirradiated parts of the nucleus (Figure 6B, 30 min after UV exposure). In contrast to XPG, the ERCC1 protein was not recruited to damaged areas in

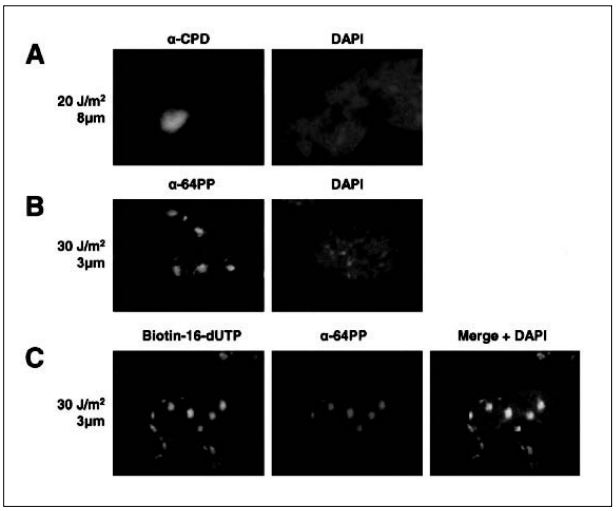


Figure 3. UV Exposure through Isopore Polycarbonate Filters Causes Locally Damaged Areas in the Nuclei
Normal human fibroblasts (VH25) were UV-irradiated with 20 or 30 J/m^2 through a 3 or 8 μm pore filter and immediately fixed. Immunofluorescent labeling was performed using (A) an antibody against CPD (α-CPD) or (B) an antibody against 6-4PP (α-64PP).
In addition (C), VH25 cells were locally exposed to UV radiation, incubated for 20 min in culture medium, and permeabilized, after which run-on DNA synthesis in the presence of biotin-dUTP was carried out. Cells were subsequently immunolabeled for both DNA repair synthesis (biotin-16-dUTP) and the presence of DNA damage, i.e., 6-4PP (α-64PP). See the Appendix for a colour version of this figure.

XP-A fibroblasts (Figure 6B), indicating that XPA protein is required for the assembly of the 5′ endonuclease XPF-ERCC1 in the incision complex but that the 3′ endonuclease XPG can associate with the complex independent of functional XPA protein. Interestingly, the accumulated quantities of TFIIH, XPC, and XPG proteins in damage spots in XP-A cells did not diminish in time and were still present 24 hr after UV exposure (data not shown), indicating that the complex must be rather stable. Alternatively, an equilibrium between NER proteins complexed at the site of damage and free-moving NER factors might account for the results.

Unlike XP-A cells, locally UV-irradiated XP-C fibroblasts did not exhibit an accumulation of XPG at damage spots. Moreover, in the absence of the XPC protein no recruitment of ERCC1 to sites of damage was discerned, suggesting that the recruitment of both structure-specific NER endonucleases is dependent on a functional XPC protein.

Binding of NER Endonucleases to the Incision Complex

Extracts from ERCC1-and XPF-deficient rodent and human cells are not capable of performing 5′ incision but can still carry out 3′ cleavage (Mu et al., 1996; Sijbers et al., 1996; Evans et al., 1997). This suggests that the entry of the XPG protein to the complex and the subsequent incision event do not require a functional ERCC1-XPF complex. To test whether or not the recruitment of endonucleases to the incision complex can take place independently of each other, we examined the distribution of XPG and ERCC1 proteins in XPF- and XPG-deficient cell strains. XP24KY (XP-F) cells are sensitive to UV light and severely defective in repair of 6-4PP and CPD (A.V.H., unpublished data). Figure 6 shows that, in locally irradiated XP-F cells, the XPG protein accumulated at a damage spot when cells were fixed and immunolabeled 30 min after UV irradiation. Also, the recruitment of the XPA protein to the UV damage was unaffected by the absence of functional XPF protein. Complementary to these experiments, we examined the distribution of ERCC1 in XPCS1RO (XP-G) cells derived from a patient with severe clinical XP and characteristics of CS (Nouspikel et al., 1997). In these cells, ERCC1 and XPA were found to be localized at spots of DNA damage. These data suggest that XPG and ERCC1-XPF are recruited to DNA damage independent of each other and that XPA can associate with the complex without the simultaneous presence of the functional 5′ and 3′ NER endonucleases.

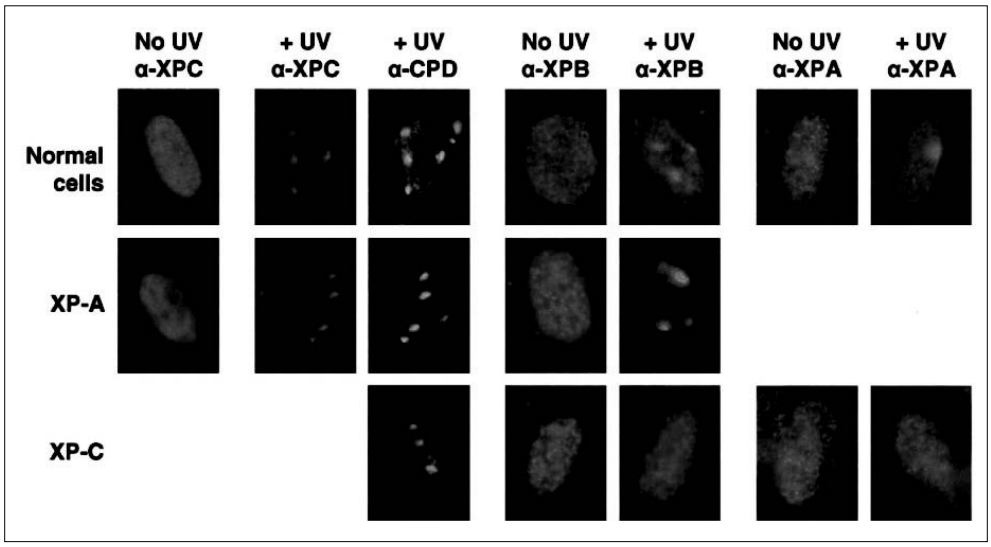


Figure 4. Recruitment of NER Proteins to Sites of UV Damage
Normal human (VH25), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were UV exposed to 30 J/m^2 through 3 μm filters or mock irradiated and at 15 min after exposure immunolabeled with an antibody (red) against XPC and an antibody (green) against CPD or labeled with antibodies against XPB or XPA. See the Appendix for a colour version of this figure.

Discussion

XPC-hHR23B Is the DNA Damage Sensor in Global Genome NER

The protein complexes XPC-hHR23B and XPA-RPA have been proposed to play a key role in DNA damage recognition. However, biochemical studies have led to contradictory results with respect to the order of assembly of these proteins in the NER complex. Here, we provide direct evidence that XPC-hHR23B is the principal DNA damage binding protein essential for the recruitment of NER components to the site of DNA damage and that the complex functions at one of the very first stages of lesion identification. This is based on the observation that, in normal human cells, both XPA and XPC concentrate at sites of DNA damage; however, while the recruitment of XPA to DNA damage requires functional XPC, XPA is not needed for the accumulation of XPC at sites of DNA damage. Our results fully support the model proposed by Sugasawa et al. (1998). Based on repair of NA-AAF adducted DNA in a competitive NER assay, these authors concluded that XPC-hHR23B is the initiator of global genome NER acting before the XPA protein. In addition, the much greater preference of XPC-hHR23B for UV-damaged DNA than for the XPA protein recently reported by Batty et al. (2000) is consistent with this idea. Our results are inconsistent with the model proposed by Wakasugi and Sancar (1999) in which RPA-XPA-DNA ternary complexes are formed first at the site of DNA lesions to initiate NER. As discussed in detail by Batty et al. (2000), experimental conditions used in in vitro reactions rather than mechanistic reasons most likely account for different results of the biochemical assays.

Assembly of the Preincision Complex: Recruitment of TFIIH to DNA Damage Relies on Functional XPC Protein

Once bound to a DNA lesion, XPC-hHR23B may induce conformational changes to the DNA helix that attract or facilitate the loading of other NER components, including the transcription factor TFIIH (Sugasawa et al., 1998). This factor is, apart from its role in basal transcription, a vital component of NER required for opening of the DNA helix at the vicinity of the lesion (Drapkin et al., 1994; Feaver et al., 1993; Schaeffer et al., 1993). Local damage induction in confluent cells resulted in a strong TFIIH signal at the sites of damage and a clear diminution of the fluorescence level outside the irradiated spots. This indicates that TFIIH is mobile and recruited from regions in the nucleus distal from the sites of DNA damage. Hence, the increase in fluorescent signal in confluent cells globally exposed to UV might be due to recruitment of TFIIH from putative storage sites for assembly of the NER complex.

The finding that the recruitment of TFIIH to sites of DNA damage occurs in XP-A cells but not in XP-C cells strongly suggests that in vivo TFIIH is recruited to DNA lesions by the XPC-hHR23B protein complex without the requirement of XPA. Previous biochemical experiments have generated conflicting results with regard to associations between XPC-hHR23B and TFIIH. Copurification of XPC with TFIIH has been reported for mammalian cells (Drapkin et al., 1994) as well as for the homologous proteins in yeast (Svejstrup et al., 1995), whereas other investigators failed to observe detectable quantities of a stable association between XPC-hHR23B and TFIIH in undamaged cells (van der Spek et al., 1996). Evidence supporting direct interactions between XPC-hHR23B and TFIIH in undamaged mammalian cells recently has been obtained by immunoprecipitation of XPC-hHR23B with the cyclin H component of TFIIH (Yokoi et al., 2000). Moreover, XPC-hHR23B was shown to be necessary for the efficient association of TFIIH to damaged DNA in cell extracts (Yokoi et al., 2000). In contrast, other studies have implicated a role of XPA in the recruitment of TFIIH to damaged DNA by direct interaction of XPA and TFIIH (Nocentini et al., 1997; Park et al., 1995). Our results clearly show that in vivo TFIIH requires XPC-hHR23B for its assembly in the preincision complex rather than XPA.

Recruitment of Endonucleases XPG and ERCC1-XPF

The assembly of a functional incision complex requires the binding of the structure-specific endonucleases XPG and ERCC1-XPF responsible for the 3′ and 5′ incision in NER, respectively. As shown in XP-F- and XP-G-cells, each of the two NER endonucleases is sequestered to the site of local DNA damage without requirement of a functional XPG or ERCC1-XPF partner. However, damage recruitment might require an XPG and ERCC1-XPF function distinct from their NER endonuclease activities. One of the *XPF* alleles in XP24KY codes for a truncated XPF protein, while the second allele specifies a protein with a single amino acid substitution that might still exert protein-protein interactions (Matsumura et al., 1998). XPCS1RO cells are homozygous for a single base deletion that results in a severely truncated XPG protein (Nouspikel et al., 1997). ERCC1-XPF depends on functional XPA protein for its recruitment to DNA damage consistent with the specific association of ERCC1 and XPF with XPA in vitro (Li et al., 1995a; Bessho et al., 1997). In this regard, it is also interesting to note that apparently the interaction of ERCC1-XPF with RPA (de Laat et al., 1998) is insufficient for ERCC1-XPF to be incorporated into the incision complex in the absence of XPA. Since XPA can accumulate at damage spots in the absence of functional XPF protein, the XPA protein might anchor ERCC1-XPF in the incision complex.

In contrast to ERCC1-XPF, the recruitment of XPG to the incision complex does not depend on functional XPA protein. However, its recruitment is apparently insufficient to perform 3′ incision, since UV-irradiated XP-A cells are completely devoid of incision activity. Most likely,

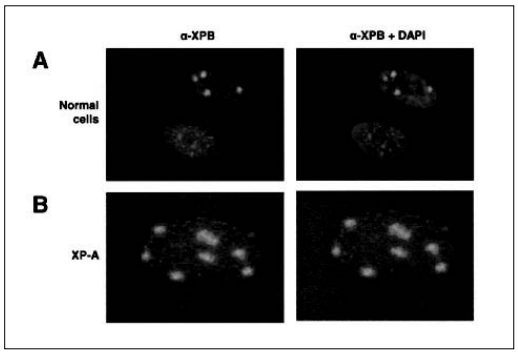


Figure 5. Reduction of XPB in Unexposed Parts of the Nucleus after Local UV Irradiation
Normal human fibroblasts (VH25) (A) or XP-A cells (XP25RO) (B) were UV irradiated with 30 J/m^2 through 3 μm pore filters and immunolabeled for XPB 15 min or 4 hr after UV exposure, respectively. See the Appendix for a colour version of this figure.

incision by XPG is activated by the XPA protein in cooperation with RPA, since the latter has been shown to be essential for the formation of dual incision in NER (Mu et al., 1996; de Laat et al., 1998).

It has been proposed (Wakasugi and Sancar, 1999) that XPC-hHR23B and XPG cannot exist in the incision complex simultaneously and that the entry of XPG to the complex coincides with XPC-hHR23B leaving the complex. Our data show that XPG, XPC-hHR23B, and TFIIH complex accumulate simultaneously in damage spots in repair-deficient XP-A cells and that this state is maintained up to 24 hr without removal of XPC. These findings either suggest that XPC and XPG can reside together in the incision complex or that complexes containing either XPC-hHR23B or XPG exist in a dynamic equilibrium at the site of the DNA lesion.

Our results indicate that NER is accomplished by sequential assembly of repair proteins rather than by a preassembled repairosome. The same conclusion was drawn from studies with a GFP tagged ERCC1 protein in mammalian cells (Houtsmuller et al., 1999).

Complex Formation Is Triggered by 6-4PP

The observed reallocation of NER factors to sites of DNA damage represents the assembly of the NER incision complex engaged in GGR rather than in TCR. This is based on the observations that XP-C cells proficient in TCR do not display a significant reallocation of TFIIH to sites of DNA damage while changes in TFIIH in globally irradiated CS-B cells (normal GGR but defective TCR) closely mimic those observed in normal cells. The time period during which TFIIH fluorescence returns to the pre-UV pattern in normal human cells closely follows the time course of repair of 6-4PP rather than of CPD (van Hoffen et al., 1995), suggesting that 6-4PP are the main stimulus for recruitment of NER proteins. Both XPA-RPA and XPC-hHR23B bind to 6-4PP (Wakasugi and Sancar, 1999; Batty et al., 2000), but XPC-hHR23B has a much higher level of affinity for 6-4PP than CPD (Batty et al., 2000). Obviously, GGR of CPD as well as TCR attract insufficient numbers of NER proteins to allow visualization of the incision complexes.

A Model for the Assembly of the Human NER Excision Complex

The assembly of the NER complex is described with a brief summary of the various steps (Figure 7).

Damage Recognition

From the two NER proteins with documented specificity for damaged DNA in vitro, i.e., XPC-hHR23B and XPA-RPA (Sugasawa et al., 1998; Wakasugi and Sancar, 1999), only XPC-hHR23B

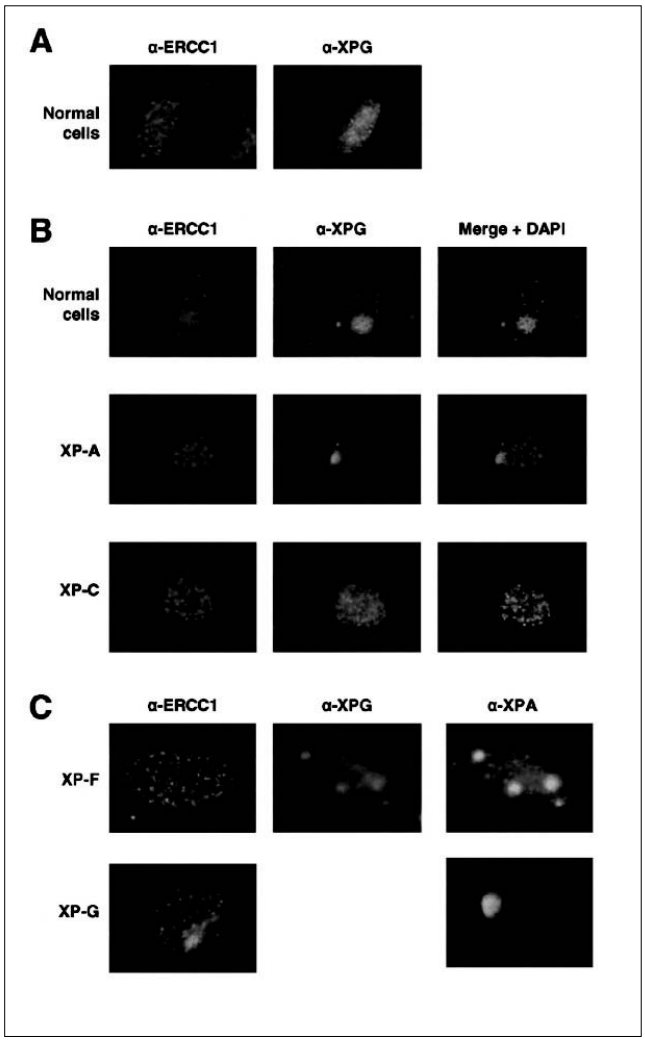


Figure 6. Recruitment of the NER Endonucleases to the Sites of UV Damage
Confluent fibroblasts were UV irradiated (30 J/m^2) through 3 µm filters or mock irradiated, fixed 15 min after UV exposure and immunolabeled for ERCC1, XPG, or XPA. (A) Unexposed normal human fibroblasts (VH25) were immunolabeled for ERCC1 and XPG. (B) Normal human (VH25), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were immunolabeled for ERCC1 and XPG. (C) XP-F (XP24KY) and XP-G (XPCS1RO) fibroblasts were immunolabeled for XPG and ERCC1, respectively, or XPA. See the Appendix for a colour version of this figure.

is the damage recognition factor for the GGR pathway in vivo. Although XPC-hHR32B might be the first protein to bind to the damage, our results do not exclude that in vivo other factors might bind to DNA damage prior to XPC-hHR23B. XPC-hHR23B might either move freely through the nucleus as demonstrated for ERCC1-XPF (Houtsmuller et al., 1999) or might be bound to chromatin (van der Spek et al., 1996), but, upon infliction of DNA damage, XPC-hHR23B will be recruited to sites of DNA lesions, even from locations distal to the damage. In the case of UV damage, the kinetics of incision complex formation strongly suggest that XPC-hHR23B binds preferentially to 6-4PP without the requirement of XPA. This is consistent with the observation that in vitro binding of XPC-hHR23B to 6-4PP occurs in the absence of XPA-RPA (Sugasawa et al., 1998; Yokoi et al., 2000; Batty et al., 2000).

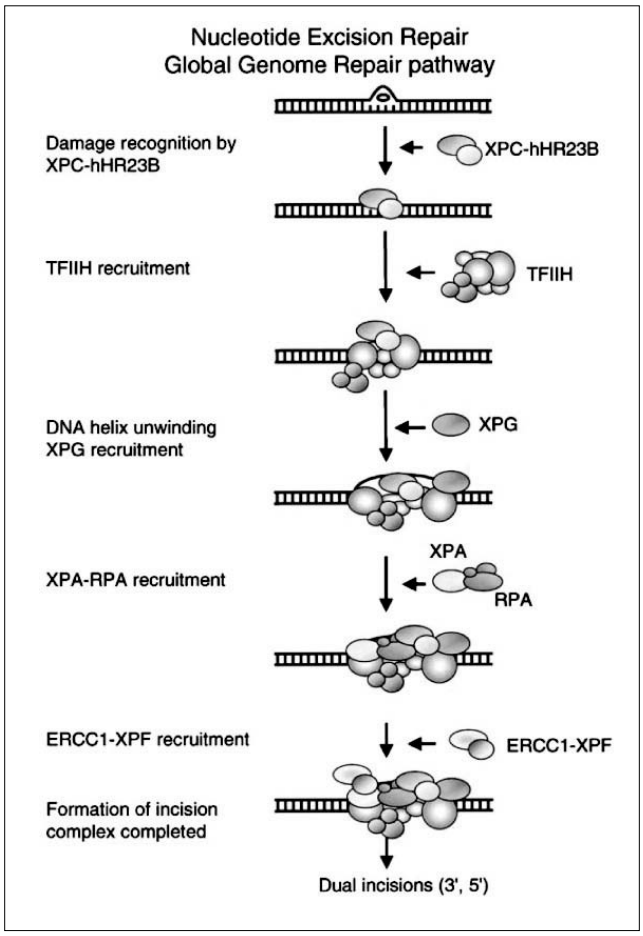


Figure 7. Model for the Assembly of the Human NER Incision Complex

Formation of the Incision Complex

Conformational changes in DNA induced by XPC-hHR23B (Sugasawa et al., 1998; 2001) could favor the subsequent binding of other NER factors such as TFIIH. Recruitment of TFIIH requires XPC-hHR23B but occurs independently of XPA, thereby generating a ternary complex. The two NER endonucleases XPG and ERCC1-XPF can bind independently from each other. XPG endonuclease associates to the incision complex in the absence of XPA, but at this stage no 3′ cleavage takes place. The assembly of XPG in the incision complex might be mediated by its interaction with TFIIH, as several subunits of TFIIH have been shown to interact with XPG (Iyer et al., 1996). However, the order of incorporation in the incision complex is not definite. XPG may be incorporated before TFIIH. Once the incision complex consisting of XPC-hHR23B, TFIIH, and XPG (as well as RPA) has been constituted, the complex is completed by the association of ERCC1-XPF requiring XPA. The presence of XPA will not only guide ERCC1-XPF to the site of DNA damage to allow the 5′ cleavage, but also activates XPG to perform the 3′ cleavage.

Experimental Procedures

Cell Culture

The primary diploid human fibroblasts were derived from a normal individual (VH25), a Cockayne syndrome patient (CS1AN, complementation group B), and xeroderma pigmentosum patients (XP21RO, complementation group C; XP25RO, complementation group A; XP24KY, complementation group F; XPCS1RO, complementation group G, previously known as 94RD27 [Nouspikel et al., 1997]). The cells were seeded on glass coverslips coated with Alcian blue (Fluka) as described (M.J. Moné et al., submitted) and grown to confluency in Ham's F10 medium (without hypoxanthine and thymidine) supplemented with 15% fetal calf serum and antibiotics at 37°C in a 2.5% CO_2 atmosphere. For experiments aimed at examining the fluorescent signal of TFIIH (XPB) after global UV irradiation, fibroblasts were kept confluent for 8–12 days without renewal of the medium.

UV Irradiation

Prior to irradiation, medium was aspirated and kept at 37°C. Subsequently, cells were rinsed with PBS and exposed to global UV irradiation with a Philips TUV lamp (predominantly 254 nm) at a dose rate of ~0.14 J/m^2/s. For local UV irradiation, the cells on coverslips were covered with an isopore polycarbonate filter with pores of 3 μm or 8 μm diameter (Millipore, Badford, MA) during UV irradiation with the Philips TUV lamp as described (M.J. Moné et al., submitted). Subsequently, the filter was removed, the medium was added back to the cells, and cells were returned to culture conditions.

Antibodies

Primary antibodies (the final dilutions are indicated between parentheses) employed in this study were rabbit IgG polyclonal anti-XPC (1:500); rabbit IgG polyclonal anti-ERCC1 (1:100); mouse IgG monoclonal anti-XPB (1:5000), a gift from Dr. J-M. Egly (IGMC, Illkirch, France); rabbit IgG polyclonal anti-XPA (1:1000), a gift from Dr. K. Tanaka (Osaka University, Japan); mouse IgG monoclonal anti-CPD (TDM2) and anti-64PP (64 M2) (1:2000 and 1:200, respectively), gifts from Dr. O. Nikaido (Kanazawa University, Kanazawa, Japan); and mouse IgG monoclonal anti-XPG (1:100), a gift from Dr. R. Wood (ICRF, London,UK). Rabbit IgG polyclonal anti-XPB (1:200) and rat IgG monoclonal anti-bromodeoxyuridine (1:100) were obtained from Santa Cruz Biotechnology and from Harlan Sera-Lab (Sussex, UK), respectively. Secondary antibodies utilized in this study were sheep anti-mouse IgG; Texas red-conjugated donkey anti-sheep IgG; Texas red-and FITC-conjugated donkey anti-mouse IgG; Cy3-con-jugated goat anti-rabbit IgG; Cy3-conjugated mouse anti-rabbit IgG plus IgM; FITC-conjugated avidin; and horseradish peroxidase-con-jugated goat anti-mouse IgG. All secondary antibodies were obtained from Jackson Laboratories (Westgrove, PA) and were used according to the manufacturer's instructions.

Fluorescent Labeling

Cells were washed twice with cold PBS and subsequently fixed and lysed by addition of PBS containing 2% formaldehyde and 0.2% Triton X-100 (Fluka) while maintaining the cells on ice for 15 min. Cells were washed twice with cold PBS and incubated with 3% bovine albumin (Sigma) in PBS for 30 min at room temperature. In order to visualize UV-induced photoproducts and bromodeoxyuri-dine-labeled DNA, cells were washed twice with PBS, treated with 2 M HCl for 5 min at 37°C to denature the DNA, and washed once with PBS. Cells were subsequently rinsed once with washing buffer (WB: PBS containing 0.5% bovine albumin [Sigma] and 0.05% tween-20 [Sigma]), incubated with the appropriate primary antibodies (diluted in WB) for 2 hr at room temperature, and washed three times with WB. Incubation with secondary antibodies (diluted in WB) was performed at room temperature for 1 hr followed by three wash steps with WB. In experiments requiring double labeling, antibodies were mixed in WB in the appropriate dilution and incubated simultaneously. After the last antibody labeling step, cells were mounted in mounting medium containing 4′-6′-diamidino-2-phenylindole (DAPI) as a DNA counter stain (Vector Laboratories, Burlingame, CA). Dur-ing the course of the experiments, we noticed that the specificity of the XPB signal could be greatly increased by the application of a modified protocol employing washing buffer without bovine albumin as well as sheep anti-mouse IgG and Texas red-conjugated donkey anti-sheep IgG as secondary and tertiary antibody, respectively.

Labeling of DNA Repair Patches with Biotin-16-dUTP
Cells grown on coverslips were UV irradiated, incubated for 30 min under standard culture conditions, and washed twice with cold physiological buffer (PB: 130 mM KCl, 10 mM KPO4 [pH 7.4], 1 mM Na2ATP Grade II [Sigma-Aldrich], 2.5 mM $MgCl_2$, and 1 mM DTT). Subsequently, Streptolysin O (Murex Diagnostics, Dartford, UK) in PB was added to the cells at a final concentration of 0.1 units/ml, and the cells were kept on ice for 30 min. Following a single wash with cold PB, permeabilization of the cells was achieved by shifting the temperature to 37°C and by maintaining the cells at this temperature for 5 min as described previously (Jackson et al., 1994; Bouayadi et al., 1997). Permeabilized cells were washed once with cold PB, and run-on DNA synthesis was carried out by incubating the cells in PB containing dATP, dCTP, and dGTP (GIBCO-BRL) at 1 mM each, 0.5 mM biotin-16-dUTP (Boehringer Mannheim), 50 mM KPO 4 (pH 7.4), and 3.5 mM $MgCl_2$ (equimolar to the nucleotides) for 25 min at 37°C. Subsequently, cells were washed three times with cold PB, and fluorescent labeling of DNA damage was performed as described above. The biotin tag was labeled with avidin-FITC in the secondary antibody labeling step.

Microscopy
Fluorescence images were obtained with a Zeiss Axioplan 2 fluorescence microscope equipped with an AttoArc HBO 100W adjustable mercury arc lamp and fitted with appropriate filters for FITC, DAPI, Cy3, and Cy3.5/Texas red. Digital images were captured with a cooled CCD camera (Hamamatsu C5935, Hamamatsu, Japan) and processed with the Metasystems ISIS software package (Metasystems, Altlussheim, Germany). To quantitate the fluorescence signals, 30 cells were randomly chosen from each sample, and the intensity of the fluorescent signal in the nucleus was measured and expressed in arbitrary units using the ISIS software.

Western Blot Analysis
Confluent cells grown in a P90 plastic culture dish were washed twice with cold PB, covered with PB containing 0.5% Triton, and kept on ice for 15 min. Cells were detached with a rubber policeman, centrifuged, and dissolved in Laemmli buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 100 mM TrisHCl [pH 6.8], and bromophenol blue). Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membrane using standard wet electroblotting procedures. Aspecific sites were blocked with sterilized skimmed milk containing 0.05% Tween-20, and the membrane was probed with monoclonal antibodies against the TFIIH subunit XPB and subsequently with horseradish peroxidase-conjugated goat anti-mouse antibody. Finally, the membrane was incubated for 10 min in Supersignal West Dura solution (Pierce) and exposed to X-ray film (Kodak X-Omat).

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Chapter 7

Local UV-induced DNA damage in cell nuclei results in local transcription inhibition

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7 Local UV-induced DNA damage in cell nuclei results in local transcription inhibition

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Summary

UV-induced DNA damage causes cells to repress RNA synthesis and to initiate nucleotide excision repair (NER). NER and transcription are intimately linked processes. Evidence has been presented that, in addition to damaged genes, undamaged loci are transcriptionally inhibited. We investigated whether RNA synthesis from undamaged genes is affected by the presence of UV damage elsewhere in the same nucleus, using a novel technique to UV irradiate only part of a nucleus. We show that the basal transcription/repair factor TFIIH is recruited to the damaged nuclear area, partially depleting the undamaged nuclear area. Remarkably, this sequestration has no effect on RNA synthesis. This result was obtained for cells that are able to carry out NER and for cells deficient in NER. We conclude that cross talk between NER and transcription occurs only over short distances in nuclei of living cells.

Introduction

Short-wave UV light induces intrastrand DNA cross-links between adjacent pyrimidines, giving rise to the cyclobutane pyrimidine dimer (CPD) and the (6-4)photoproduct (6-4PP). These photoproducts are potent inhibitors of transcription by RNA polymerase II (RNA pol II). The removal of these DNA lesions is somehow prioritized at the expense of ongoing transcription. This may reflect an active cellular response to circumvent, for instance, the potential risks that might arise due to the production of aberrant gene products. The molecular mechanism underlying inhibition and recovery of transcription after DNA damage is not understood well (Friedberg et al., 1995).

Nucleotide excision repair (NER) is a sophisticated DNA repair mechanism that enables cells to resolve a wide variety of structurally diverse DNA injuries, including UV-induced photoproducts. NER involves the action of some 30 different gene products. Many protein interactions and biochemical characteristics of this repair system have been established (De Laat et al., 1999). There appear to be at least two mechanistically distinct NER pathways, commonly referred to as global genome repair (GGR) and transcription-coupled repair (TCR). In GGR, the XPC–hHR23B complex recognizes the damaged DNA and subsequently recruits the repair machinery to process the lesion (Sugasawa et al., 1998). On the other hand in TCR, DNA lesions are detected via an unknown mechanism that depends on RNA pol II-driven transcription (Hanawalt, 2000). Both CPD and 6-4PP, if present in the template strand of a transcribed gene, very efficiently block

elongating RNA pol II. The stalled polymerase itself probably functions as a signal for the NER factors to be recruited. In agreement, XPC–hHR23B is not involved in TCR (Van Hoffen et al., 1995), whereas at least two gene products with poorly understood molecular roles, the CSA and CSB proteins, are necessary for TCR but not for GGR (Venema et al., 1990).

In addition to the ability of UV photoproducts to pose a physical block for RNA pol II, there have been other observations that suggest a tight coupling between NER and transcription. The most striking example of such a link is the finding that the basal transcription factor TFIIH is required for NER, both GGR and TCR (Hoeijmakers et al., 1996). In NER-proficient yeast extracts, it was found that transcription of an undamaged plasmid was decreased in the presence of another UV-damaged plasmid. This inhibition was dependent on the presence of the yeast homologue of the human CSB protein, Rad26, and could be reversed by addition of TFIIH (You et al., 1998). This supports a straightforward model for transcription inhibition by DNA damage: upon DNA damage, TFIIH is recruited to sites of damage and, as a result, is not then available for transcription initiation, causing a decrease in overall transcriptional activity.

In order to understand the mechanism by which DNA damage inhibits RNA pol II-driven transcription, we have investigated whether RNA synthesis from undamaged genes is affected by the presence of UV damage elsewhere in the nucleus. We developed a simple technique that enables one to irradiate only a limited volume of cell nuclei with UV light. This allowed us to analyse the functional cross talk between NER and transcription in the intact cell, showing that both systems only interact over short distances in the nucleus.

Results and discussion

Induction and detection of local UV damage

We have developed a method that enabled us to UV irradiate a confined area of individual cell nuclei. A polycarbonate filter containing pores of defined size (e.g. 3, 5, 8 or 10 μm in diameter) was used. Cells that were grown on coverslips were briefly rinsed with phosphate-buffered saline (PBS) and after removal of excess buffer the filter was carefully positioned on top of the cell monolayer. Filter-covered cells were immediately UV-irradiated from above, after which the filter was removed. UV damage was then visualized by immunofluorescence labelling using the damage-specific monoclonal antibody TDM-2 (Mori et al., 1991). Figure 1A displays cells that were locally irradiated and subsequently immunostained for CPDs. This reveals DNA damage in discrete parts of cell nuclei. The number of nuclei that were locally damaged was consistent with the pore density of the filter (4×10^5 pores/cm^2), resulting in a cell monolayer in which most nuclei were either unirradiated or locally damaged in a single spot. A small number of nuclei contained more than one damaged nuclear area. The diameter of UV-damaged spots was consistent with the diameter of the pores. Labelling in unirradiated parts of locally damaged nuclei was indistinguishable from background levels of unirradiated control cells in a separate experiment, indicating that the filter material efficiently absorbs 254 nm UV light. This technique allows one to locally UV irradiate large numbers of cells, while unirradiated control nuclei are present in the same monolayer of cells.

TFIIH accumulates at sites of UV damage

TFIIH is indispensable for both transcription initiation and NER. Logically, this dual functionality has led to the suggestion that TFIIH could play a key role in orchestrating the down-regulation of transcription upon DNA damage-triggered onset of NER (Van Oosterwijk et al., 1996;

Mullenders, 1998; You et al., 1998). Previous experiments by Houtsmuller et al. (1999) have shown that the ERCC1/XPF heterodimer, an endonuclease that makes the 5′ incision during NER, moves freely through undamaged cell nuclei and becomes immobilized for several minutes upon induction of UV damage. This shows that at least some NER factors are likely to be recruited to damaged sites in a distributive manner, rather than by processive scanning of large genomic segments. From that notion it is possible that TFIIH is targeted to sites of DNA damage in a similar fashion. We have tested whether this is the case.

Primary fibroblasts were locally UV-irradiated and cultured for 1 h prior to fixation. Indeed, TFIIH had accumulated in large nuclear areas (Figure 1B). The size and number of these p62-enriched areas were consistent with filter specifications, and were also confirmed by applying filters with different pore size and pore density (data not shown). Moreover, double labelling shows that local UV damage and TFIIH indeed colocalize (Figure 1C). In addition to TFIIH accumulation in irradiated areas, TFIIH labelling intensity appeared to be partly depleted in undamaged parts of cell nuclei. This was the case even if a quarter to a third of the nuclear volume had been irradiated with 100 J/m^2, resulting in an estimate number of lesions per nucleus that is ~3-fold higher than the amount of lesions known to saturate the NER process and to inhibit >80% of transcription in cells that are uniformly UV-irradiated (Mayne and Lehmann, 1982). These data show that recruitment of TFIIH from undamaged to damaged nuclear areas occurs and might thus affect transcription in undamaged genomic regions.

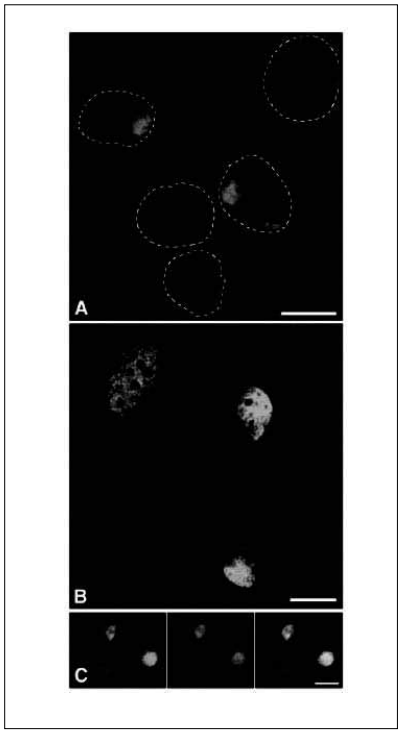

Figure 1
(A) Detection of locally induced UV damage in cell nuclei. A UV-blocking polycarbonate filter containing pores of 5 µm in diameter was used to cover a monolayer of cells. The filter-covered cells were UV-irradiated with 30 J/m^2 and CPDs were subsequently detected by immunofluorescent labelling. Dotted lines denote the contours of individual cell nuclei. Two nuclei show labelling of CPDs in discrete nuclear areas.
(B) Effect of local nuclear UV damage on the distribution of TFIIH. Human primary fibroblasts were locally UV-irradiated with 100 J/m^2 UV light, using a filter with 10 µm pores. Following irradiation, cells were grown for 1 h and immunolabelled against the p62 subunit of TFIIH. The top-left nucleus displays the characteristic labelling pattern of TFIIH in unirradiated cells, whereas the two remaining nuclei exhibit a TFIIH accumulation in UV-damaged nuclear areas, and a reduction in TFIIH signal outside these areas. A single confocal optical section is shown.
(C) Colocalization of TFIIH (green) and CPDs (red). Human primary fibroblasts were locally irradiated with 30 J/m^2 UV light, using a filter with 8 µm pores. Following irradiation, cells were grown for 30 min and dual labelled against both CPDs and the p62 subunit of TFIIH.
Bars represent 10 µm. See the Appendix for a colour version of this figure.

Local inhibition of transcription by local UV damage

In an *in vitro* yeast system, RNA pol II transcription of an undamaged template was significantly inhibited when NER became activated. Furthermore, this transcription inhibition could be restored by increasing the TFIIH concentration (You et al., 1998). Competition assays from human cell-free extracts have generated ambiguous results. Satoh and Hanawalt (1996) found no communication between transcription and NER in the presence of DNA damage. On the other hand, Vichi et al. (1997) did find damage-induced inhibition of transcription from an undamaged template, although the authors concluded that this coincided with sequestration of TATA box-binding protein rather than of TFIIH. We investigated whether local nuclear UV damage interfered with transcription in undamaged areas within the same intact nucleus.

NER-proficient human primary fibroblasts were locally irradiated with 50 J/m^2 UV light. Such a dose on a quarter to a third of the nuclear volume will generate a total number of lesions that is 30–100% larger than the number of lesions that is induced by 8–10 J/m^2 uniform UV irradiation of cells. The latter condition is known to obliterate >80% of transcription 1 h after UV irradiation. After post-irradiation culturing for 1 h, cells were allowed to incorporate BrUTP into nascent RNA during run-on transcription (Jackson et al., 1993; Wansink et al., 1993). Subsequently, they were immunofluorescently dual labelled using anti-BrdU and anti-CPD antibodies. RNA synthesis was almost completely abolished within the UV-damaged nuclear area (Figure 2, WT). Surprisingly, cells maintained normal levels of transcriptional activity throughout undamaged regions of the nucleus. Inhibition of transcription in locally irradiated nuclei was fully confined to the UV-damaged area. The BrU signal outside the irradiated spot was comparable to RNA labelling intensities of nearby unirradiated control cells. If primary fibroblasts were allowed to grow for 12 h after local UV irradiation, CPD labelling was still clearly detectable (Figure 2, WT), but cells had largely restored transcription inside the damaged area. In accordance with previous data, it is anticipated that at this point in time the NER machinery will have repaired 6-4PPs and active genes. The bulk of the remaining damage will be CPDs in inactive chromatin, as these are known to be repaired much slower (Van Hoffen et al., 1995). Our results show that UV-induced DNA damage has a short-range inhibitory effect on transcription.

As NER and transcription are intimately linked processes, we investigated the effects of local UV irradiation in NER-deficient cell lines. XP-A cells are deficient in performing NER. Although RNA synthesis was efficiently inhibited in the damaged spot 1 h after local irradiation, BrU labelling intensities in undamaged nuclear compartments were comparable to labelling intensities of undamaged cells (Figure 2, XP-A). Twelve hours after induction of local damage, XP-A cells still exhibited high levels of transcription in undamaged nuclear areas, comparable to labelling intensities of undamaged control cells. Not surprisingly, due to the absence of NER in these cells, transcription in the damaged spot remained inhibited. Taken together, our results show that there is no long-distance functional cross talk between NER and transcription. Furthermore, cells harbour sufficient TFIIH to support normal levels of transcription and high levels of NER simultaneously.

We also examined the transcription response in XP-C and CS cells, deficient in GGR and TCR, respectively. The XPC protein is the earliest damage recognition factor during GGR (Sugasawa et al., 1998; Volker et al., 2001) and hence, the bulk of genomic DNA damage will remain devoid of NER complexes. Lesions are therefore likely to be available as a decoy for damage-binding transcription factors like TATA box-binding protein. Nonetheless, 1 h after local UV irradiation, transcription inhibition remained confined to the damaged nuclear area in XP-C cells (Figure 2, XP-C). The observation that XP-C cells reinitiated RNA synthesis in the UV-damaged area

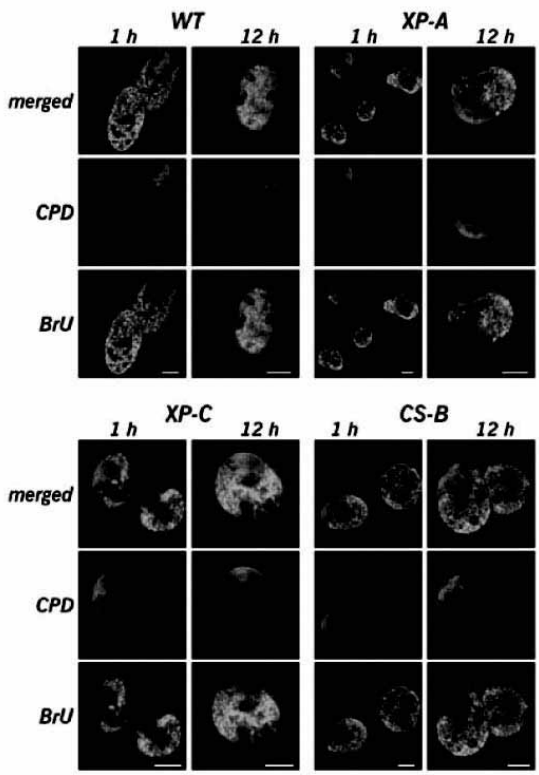

Figure 2 Effect of local nuclear UV damage on transcription
Normal human primary fibroblasts (WT; NER-proficient) and immortalized primary fibroblasts from patients suffering from xeroderma pigmentosum group A (XP-A; no NER), group C (XP-C; no GGR) or Cockayne syndrome group B (CS-B; no TCR) were studied. Exponentially growing cells were locally UV-irradiated with 50 J/m2 using filters containing 10 µm pores. After irradiation, cells were cultured for either 1 or 12 h. Subsequently, cells were allowed to incorporate BrUTP into nascent RNA during run-on transcription labelling. Nascent RNA (green) and CPDs (red) were dual labelled by immunostaining.
Single confocal optical sections are shown. Bars represent 5 µm. See the Appendix for a colour version of this figure.

12 h after local irradiation (Figure 2, XP-C) is consistent with the known ability of XP-C cells to recover inhibited transcription after UV damage (Mayne and Lehmann, 1982). CS cells are impaired in performing TCR (Venema et al., 1990) and are unable to recover transcription after inhibition by UV damage. Also in CS-B cells, BrU incorporation was not decreased in undamaged genomic regions after both 1 and 12 h of post-irradiation culturing (Figure 2, CS-B). As one would expect, transcription remained inhibited within the damaged spot.

Mechanism of UV damage-induced transcription inhibition
Several studies indicate that transcription from undamaged genes is affected by the presence of DNA damage elsewhere in the nucleus. For instance, mutations in the XPD subunit of TFIIH can lead to the repair disorder XP (XP-D cells), or may result in XP combined with CS clinical features (XP-D/CS cells). Both XP-D and XP-D/CS cells appear equally inefficient in eliminating UV damage from transcribed genes, yet they differ in their ability to recover their UV-inhibited RNA synthesis. Whereas XP-D cells are able to regain normal transcription after irradiation with 2 J/m^2 UVC, XP-D/CS cells fail to do so (Van Hoffen et al., 1999). XP-D/CS cells, despite their inefficient photoproduct removal, generate repair incisions with almost the same efficiency as normal cells (Berneburg et al., 2000). The incisions appear to be uncoupled from the NER process, as introduction of a UV-damaged plasmid in XP-D/CS cells triggered the induction of

incisions in undamaged genomic DNA. Whatever the mechanism may be, these data indicate that XP-D/CS cells sense DNA damage and respond to it *in trans*. Our results show unambiguously that inhibition of RNA synthesis by UV irradiation occurs only in the close vicinity of damaged DNA, in both wild-type and CS cells (Figure 2). Stalling of elongating RNA pol II at lesions in transcribed templates will certainly contribute to the observed transcription inhibition. However, one cannot rule out that short-range sensing of DNA damage impedes transcription of a nearby undamaged gene, suggesting a local *trans* effect. It has been shown that small numbers of RNA polymerases are tightly associated in transcription factories (Cook, 1999), suggesting some functional interaction between these polymerases. Hence, it is conceivable that stalling of for example one polymerase on a lesion could inhibit several polymerases within the same factory.

Methods

Cell culture

Primary human fibroblasts (VH25) were grown in Ham's F-10 medium containing 12% fetal calf serum (FCS), 1% glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Cells were cultured in a 5% CO2 atmosphere at 37°C.

SV-40 immortalized primary fibroblasts (gifts from Dr W. Vermeulen) XP20MA (XP-C), XP12RO (XP-A) and CS1AN (CS-B) were grown in a 1:1 mixture of Ham's F-10 and Dulbecco's modified Eagle's medium containing 10% FCS. Conditions and supplements were the same as for VH25. Cells were used at 50–80% confluency.

Local UV irradiation

Cells were rinsed in PBS. The PBS was then removed, leaving only a thin layer of buffer on top of the coverslip. An Isopore polycarbonate membrane filter (Millipore) containing pores of either 5 µm (4×10^5 pores/cm^2) or 10 µm (10^5 pores/cm^2) in diameter was placed on top of the cells. The coverslip with filter was irradiated from above using a TUV 15 W lamp (Philips) at a UVC fluency of 1.0 W/m^2, as measured at 254 nm with an SHD 240/W detector connected to an IL 1700 radiometer (International Light). The filter was then removed and cells were either fixed or cultured for another period of time. Immunofluorescent labelling. Nascent RNA was labelled with BrUTP during run-on transcription as described by Wansink et al. (1993).

Cells were fixed with 4% formaldehyde in PBS for 15 min at 4°C, permeabilized in 0.5% Triton X-100 (Serva) in PBS for 5 min, and incubated with 100 mM glycine in PBS for 10 min. Cells were rinsed in PB (130 mM KCl, 10 mM Na2HPO4 and 2.5 mM $MgCl_2$ pH 7.4) and equilibrated in WB [PB containing 0.5% bovine serum albumen (BSA), 0.2% gelatin and 0.05% Tween 20; Sigma-Aldrich]. Antibody steps and washes were in WB. BrU-labelled RNA was labelled by a rat monoclonal anti-BrdU antibody (Seralab), and detected by FITCconjugated donkey anti-rat antibody (Jackson ImmunoResearch Laboratories). TFIIH labelling was carried out using a mouse mAb against the p62 subunit of TFIIH (a gift from Dr J.M. Egly), and detection was by sheep anti-mouse Ig coupled to biotin, and FITC-conjugated streptavidin (Jackson).

Immunolabelling of CPDs was performed using mouse mAb TDM-2 (Mori et al., 1991). For this, the above steps were repeated, but prior to labelling DNA was denatured with 2 M HCl for 30 min at 37°C and blocked in 10% BSA in PB for 15 min. Detection was done using sheep anti-mouse Ig coupled to biotin (Amersham Pharmacia Biotech) and Cy3-conjugated streptavidin (Jackson). For double-labelling CPDs and TFIIH, a polyclonal anti-p62 antibody (Santa Cruz) was used, detected by goat anti-rabbit IgG coupled to FITC (Jackson). Samples were mounted in Vectashield.

Microscopy

Fluorescence microscopy was performed on a Leitz Aristoplan fluorescence microscope with a Zeiss Plan-Neofluar 63×/1.25 NA oil immersion objective and equipped with a CCD camera (Apogee).

Optical sections were recorded on an LSM510 confocal laser scanning microscope with a Zeiss Plan-Neofluar 100×/1.3 NA oil immersion objective. FITC and Cy3 were excited at 488 and 543 nm, respectively.

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Chapter 8

Xeroderma pigmentosum group A protein loads as a separate factor onto DNA lesions

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8 Xeroderma pigmentosum group A protein loads as a separate factor onto DNA lesions

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Abstract

Nucleotide excision repair (NER) is the main DNA repair pathway in mammals for removal of UV-induced lesions. NER involves the concerted action of more than 25 polypeptides in a coordinated fashion. The xeroderma pigmentosum group A protein (XPA) has been suggested to function as a central organizer and damage verifier in NER. How XPA reaches DNA lesions and how the protein is distributed in time and space in living cells are unknown. Here we studied XPA in vivo by using a cell line stably expressing physiological levels of functional XPA fused to green fluorescent protein and by applying quantitative fluorescence microscopy. The majority of XPA moves rapidly through the nucleoplasm with a diffusion rate different from those of other NER factors tested, arguing against a preassembled XPA-containing NER complex. DNA damage induced a transient, ~5-min immobilization of maximally 30% of XPA. Immobilization depends on XPC, indicating that XPA is not the initial lesion recognition protein in vivo. Moreover, loading of replication protein A on NER lesions was not dependent on XPA. Thus, XPA participates in NER by incorporation of free diffusing molecules in XPC-dependent NER-DNA complexes. This study supports a model for a rapid consecutive assembly of free NER factors, and a relatively slow simultaneous disassembly, after repair.

Introduction

DNA-damaging agents continuously challenge the integrity of DNA. DNA lesions directly affect transcription and replication, leading to cell death and contributing to aging, and also induce mutations that eventually cause carcinogenesis (13). Various repair mechanisms have evolved to prevent the consequences of DNA injuries and to preserve genetic integrity (21, 27). In mammals, the nucleotide excision repair (NER) process is the most important repair pathway for removal of UV light-induced lesions, including cyclobutane pyrimidine dimers, CPDs and 6-4 photoproducts and a wide range of helix-distorting chemical adducts. The significance of a functional NER system is apparent from the severe clinical features expressed by individuals suffering from the hereditary disorders xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (7). Patients suffering from the prototype repair disorder XP are extremely sensitive to solar (UV) exposure, have an increased risk for skin cancer, and frequently exhibit neurological symptoms.

Detailed biochemical studies have shown that >25 polypeptides are required for in vitro NER (4, 15, 38). Two distinct NER subpathways operate within mammals, transcription-coupled repair (TCR) and global genome repair (GGR), each addressing a specific genome compartment and category of damages (10, 25). The distinction between these subpathways originates from the first steps of the mechanism, i.e., lesion detection. Lesions that block RNA polymerase II transcription elongation are preferentially repaired by TCR and require the CSA, CSB, and XAB2 proteins (45). TCR allows rapid resumption of the vital process of RNA synthesis and is particularly important for lesions that are inefficiently repaired by GGR-NER, such as CPDs. Injuries anywhere in the genome are targeted by the slower operating GGR. Damage sensing in this process is performed by the XPC/hHR23B/centrin 2 heterotrimeric complex (2, 52, 53, 60). In addition, the DNA damage binding, UV-DDB protein complex (9, 33) helps to identify CPDs in GGR (31, 35, 53, 56). On the other hand, a complex consisting of XPA and the single-stranded DNA binding protein RPA (replication protein A) (37) has been suggested to be the primary lesion detector in GGR (61), but this finding was recently challenged by Reardon and Sancar, who claimed that only RPA is the initial damage sensor (48).

The next step in NER is performed by the nine-subunit TFIIH complex (60, 64), containing the XPB and XPD helicases. TFIIH locally opens the DNA double helix around the lesion (20, 22), likely in the presence of XPG. Subsequently, XPA and RPA play an essential but as yet not fully understood role in the core of the reaction. XPA and RPA are necessary for further assembly and proper orientation of the incision proteins ERCC1/XPF and XPG (14). The latter are structure-specific endonucleases incising the damaged strand around the lesion (5′ and 3′, respectively), leaving an excised stretch of ~30 nucleotides. DNA polymerase δ/ε and auxiliary factors fill the remaining gap, which is sealed by ligase 1 (15, 27, 38).

Despite detailed knowledge of the in vitro NER mechanism, little is known about how this process operates in living cells. Different models for the organization of NER have been proposed, ranging from an ordered assembly of factors (1, 38, 44, 60) or four defined subcomplexes (23, 48, 61) to a preassembled NER holocomplex (54). Recently, our group provided evidence that some of the NER constituents roam through the nucleoplasm by diffusion and are transiently bound to complexes actively engaged in NER (28, 29). The XPA protein plays a crucial role within NER, since in the absence of this protein NER is completely abolished. Multiple interactions of XPA with other NER factors have been reported, suggesting a central role in complex assembly (14). These include the XPA/RPA complex exhibiting a higher specificity and affinity for damaged DNA than XPA alone and the ERCC1/XPF/XPA ternary complex, as well as an association with TFIIH (summarized in references 5 and 15). In addition, a link between XPA and TCR was suggested by the observed associations of CSB with the core NER factors XPA, XPG, and TFIIH (32, 51) and between XPA and XAB2 (45).

To provide further insight into the molecular interactions of XPA in living cells, we tagged this central NER factor with the green fluorescent protein (GFP) and studied its distribution and mobility in living cells. We applied fluorescence-based imaging and bleaching (fluorescence redistribution after photobleaching [FRAP]) methods using confocal laser scanning microscopy (29, 30) in cells containing local UV damage (43, 60). In similar previous studies of the NER factors ERCC1 and TFIIH (28, 29) our group provided evidence that at least these proteins do not reside in large NER holocomplexes. Here we present our findings on the dynamic properties of XPA-GFP, NER reaction kinetics, and the mode of complex assembly in living cells.

Materials and methods

Cell lines

Cell lines used in this study were the simian virus 40 (SV40)-immortalized fibroblasts MRC5 (wild type), XP2OS (XP-A), XP12RO (XP-A), and XP20MA (XP-C). These were cultured in RPMI$^+$-HEPES medium supplemented with antibiotics and 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2. Primary fibroblasts used for microneedle injection, C5RO (wild type) and XP25RO (XP-A), and those used for immunofluorescence studies, VH25 (wild type), XP25RO (XP-A), XP21RO (XP-C), XPCS1RO (XP-G/CS), XP1DU (XP-D), XP8BR (XP-D/CS), and XP131MA (XP-B/CS), were cultured in Ham's F10 medium supplemented with antibiotics and 15% fetal calf serum.

Generation of GFP-tagged XPA

GFP-tagged XPA was generated by in-frame ligation of an XPA cDNA fragment (nucleotides 9 to 863) encoding the entire XPA, except for the first three amino acids, into pEGFP-C1 (Clontech). His_9 and hemagglutinin (HA) tags were both added to the N terminus of enhanced GFP (eGFP). The His_9-HA encoding sequence was introduced via ligation of a double-stranded oligonucleotide at the N terminus of eGFP after *Nhe*I-*Nco*I digestion (5′ CTAGCAAC ATG GGC CAC CAC CAT CAC CAT CAT CAC CAC CAC GGC **TAC CCA TAC GAT GTT CCA GAT TAC GCA AGC** GC 3′), resulting in a fusion gene under the control of a cytomegalovirus promoter encoding a 9-histidine stretch (underlined)-HA tag (bold)-eGFP–XPA hybrid polypeptide.

Microneedle injection and UDS

Microinjection of cDNA into cultured, multinucleated primary XP-A (XP25RO) fibroblasts was performed as described previously (59). After injection, cells were incubated for 24 h at 37°C in standard medium to allow expression of the cDNA. Fluorescent (GFP) images were obtained with an Olympus IX70 microscope (excitation at 455 to 490 nm band pass filter, long pass emission filter, >510 nm). DNA repair capacity was determined by measuring unscheduled DNA synthesis (UDS). Fibroblasts were UV-irradiated at 16 J/m^2 (254 nm), pulse-labeled for 2 h using [^{3}H]thymidine (20 μCi/ml), and fixed for in situ autoradiography. Autoradiographic grains above the nuclei of injected polykaryons were counted and compared with the amount of grains above the nuclei of wild-type primary fibroblasts (C5RO) treated in parallel.

Transfection of human fibroblasts

XPA-and XPC-deficient human SV40transformed fibroblasts were transfected with the His9-HA-eGFP–XPA fusion expression plasmid containing the NEO gene by using SuperFect (Qiagen). Cells were diluted 24 h after transfection, and medium containing 0.3 mg of G418, gentamicin/ml was added. Gentamicin-resistant XP-A cells were subsequently selected for UV resistance by irradiation three times, with a 1-day interval with 4 J of UV-C/m^2. Surviving clones were further selected for the presence and proper expression level of nuclear fluorescence by cell sorting using a FACS-Vantage cell sorter (Becton Dickinson). eGFP fluorescence was excited at 488 nm with a 20-mW Ar laser, and eGFP emission was detected using a 525-nm dichroic shortpass mirror and a 530/30-nm bandpass filter.

Immunoblot analysis and UV survival

Whole-cell extracts prepared by sonication were separated by sodium dodecyl sulfate–11% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Expression of the fusion gene was analyzed by hybridizing the membranes with a polyclonal anti-XPA antibody (Santa Cruz), followed by a secondary antibody (goat anti-rabbit conjugated with horseradish peroxidase [Biosource International]), and detected using enhanced chemiluminescence, Amersham.

For UV survival experiments, cells were plated and exposed to different UV doses 2 days after plating. Survival was determined 3 days after UV irradiation by incubation at 37°C with [^{3}H]thymidine pulse-labeling as described elsewhere (24).

Immunofluorescence
Cells were grown on glass coverslips and fixed with 2% paraformaldehyde at 37°C. Coverslips were washed three times for 5 min with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and subsequently washed with PBS^+ (PBS containing 0.15% glycine and 0.5% bovine serum albumin). Cells were incubated at room temperature with primary antibody for 1.5 h in a moist chamber. Subsequently, coverslips were washed three times with PBS–Triton X-100 and PBS^+, incubated for 1 h with secondary antibody at room temperature, and again washed three times in PBS–Triton X-100. Samples were embedded in Vectashield mounting medium (Vector) containing 0.1 mg of DAPI (4′-6′-diamidino-2-phenylindole)/ml. Primary antibodies used for immunolabeling were as follows: rabbit polyclonal anti-XPA antibodies (kindly provided by K. Tanaka, Osaka University, Osaka, Japan), rabbit polyclonal anti-XPC (57), mouse anti-CPD monoclonal antibodies (gift from O. Nikaido, Kanazawa University, Kanazawa, Japan), and mouse monoclonal anti-RPA70. Secondary antibodies were as follows: Alexa 594-conjugated goat anti-rabbit antiserum (Molecular Probes) and Cy2-and Cy3-conjugated goat anti-mouse antiserum (Jackson ImmunoResearch Laboratories). Fluorescent microscopy images were obtained with a Leitz Aristoplan microscope equipped with epifluorescence optics and a PLANAPO 63X, 1.40-numerical aperture oil immersion lens or with a Zeiss Axioplan 2 microscope equipped with epifluorescence optics. Quantification of fluorescence signal was determined using macrocontrolled digital image analysis software (KS-400; Zeiss).

Confocal microscopy
Digital images of GFP-expressing living cells were obtained using a Zeiss LSM 410 microscope equipped with a 60-mW Ar laser (488 nm) and a 40X, 1.3-numerical aperture oil immersion lens. Images of single nuclei were taken at a sample interval of 100 nm. For analysis of GFP-XPA expression levels, confocal planes were scanned at relatively low resolution (625-nm sample interval). A computer-controlled acousto-optic transmission filter was used to vary the intensity of the line of an Ar laser. GFP fluorescence was detected using a dichroic beamsplitter (488/543 nm) and an additional 515 to 540-nm bandpass emission filter.

UV irradiation
For total UV DNA damage induction, cultured cells were rinsed with PBS and UV-irradiated on coverslips with a Philips TUV lamp (254 nm) at a dose rate of ~0.8 $J/m^2/s$. To apply local UV damage on living fibroblasts, cells were UV-irradiated through an isopore polycarbonate filter (Millipore) containing 5 μm-diameter pores as described previously (43, 60). Subsequently, after filter removal, cells were either cultured or microscopically examined or fixed with paraformaldehyde and further processed for immunocytochemistry as described above.

FRAP and fluorescence loss in photobleaching (FLIP)
FRAP experiments were used to determine the effective diffusion coefficient (D_{eff}) of GFP-labeled XPA (under various experimental conditions) (29, 30). Briefly, a narrow strip spanning the entire nucleus was bleached for 200 ms at high laser intensity (100% of the 488-nm line of a 60-mW Ar laser). Subsequently, the recovery of fluorescence in the strip was monitored at intervals of 100 ms at 5% of the laser intensity applied for bleaching. The effective diffusion coefficient (D_{eff}) was estimated by calculating the relative fluorescence intensities given by the equation $FR_t = (I_t - I_0)/(I_\infty - I_0)$, where I_∞ is the fluorescence intensity measured after complete recovery, I_0 is the fluorescence intensity immediately after bleaching, and I_t is the fluorescence intensity measured at different time points (at 100-ms intervals). The resulting curves were fit to a theoretical diffusion model as described previously (18) (1-D diffusion). In this model, fluorescence recovery (FT) is defined by the equation $FT_t = 1 - [w^2 \times, w^2 + 4D\pi t^{-1}]^{½}$, where w is the width of the bleached strip, D is the diffusion coefficient, and t is time. The optimal fit was found by minimizing $\Sigma(FR_t - FT_t)^2$ (ordinary least squares) for both the diffusion coefficient (D) and the fluorescence intensity immediately after bleaching (I_0). The immobile fraction, FR was calculated from the equation $FR = 1 - [(I_\infty - I_0)/(I_{t<0} - I_0)] - (N_{mobile,bleached}/N_{tot})$, where N represents the number of molecules, $I_{t<0}$ and I_0 are the fluorescence intensities immediately before and after bleaching, respectively, and I_∞ is the fluorescence intensity measured after complete recovery. $N_{mobile,bleached}/N_{tot}$ is subtracted to correct for the fraction of mobile molecules in the relatively small volume of the nucleus that were bleached by the high-intensity laser pulse.

The immobilization measurements of GFP-labeled molecules were performed using a modified FRAP assay (FRAP-FIM [FRAP for immobilization measurements]) as described previously (29, 30). Using this method, quantitative fluorescence over a confocal plane of the entire nucleus was measured. Briefly, a small spot in the center of the nucleus was bleached at low laser intensity for a relatively long period (4 s at relatively low laser intensity [15% of a 60-mW Ar laser]) with the aim of bleaching a significant proportion of the GFP-tagged molecules in the nucleus. Subsequently, after an additional 4 s, a postbleach image was made and compared with a prebleach image of the same focal plane. The fluorescence intensity ratio (I_{post}/I_{bleach}) was plotted as a function of distance to the laser bleach spot, generating a fluorescence ratio profile (FRP) (see Fig. 4). Chemically immobilized molecules, paraformaldehyde fixation were used as 100% immobilization controls.

FLIP experiments were used to determine the residence time of GFP-tagged XPA molecules in local UV-irradiated areas (see above). For this, a strip at a relatively long distance from the site of local damage was photobleached for 4 s at relatively low laser intensity. Subsequently, fluorescence intensity was monitored in the local damage area, in the bleached area, and in an undamaged control region located at the same distance from the bleached area as the local damage area. The difference between the fluorescence in the damage area and that in the control region was plotted and the time at which 10% of the initial difference was reached was taken as an estimate for the residence time of individual molecules associated with the local damage area.

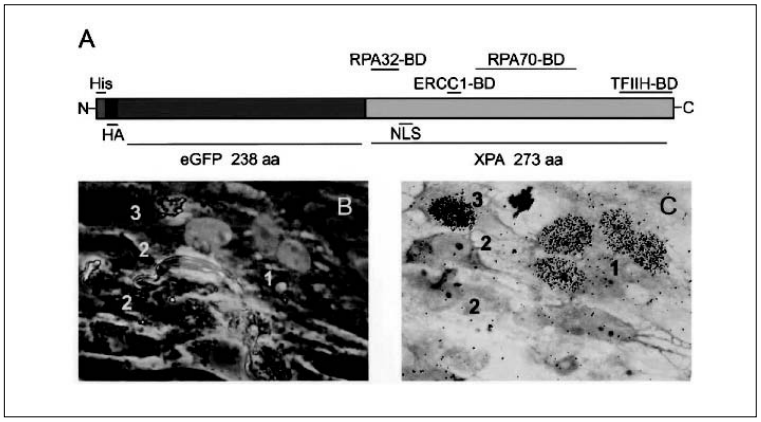

Figure 1. Functionality of GFP-XPA
(A) Schematic representation of the His9-HA-eGFP–XPA fusion gene with the different binding domains indicated. NLS, nuclear localization signal; BD, binding domain; aa, amino acids. (B) Fluorescence image of XP-A cells injected with GFP-tagged XPA cDNA. Only the multinucleated cell microinjected with GFP-XPA cDNA showed a homogeneous nuclear expression (number 1); surrounding cells were not injected (number 2). (C) Measurement of the repair capacity of cells with fluorescent nuclei by means of UV-induced UDS (see Materials and Methods). The amount of silver grains above the nuclei of the injected cells (number 1) was comparable to what was seen with wild-type cells (not shown), whereas the surrounding XP-A fibroblasts (number 2) show the low level of DNA synthesis typical for UV-exposed XP-A cells. The cell indicated with the number 3 is in S phase. See the Appendix for a colour version of this figure.

Results

Construction and characterization of GFP-XPA

The XPA cDNA was fused to the eGFP cDNA containing histidine and HA tags at its N terminus, see Materials and Methods, resulting in a His_9-HA-eGFP–XPA hybrid gene (designated GFP-XPA) (Fig. 1A). To verify that the GFP tag did not interfere with the XPA function, the fusion gene was microinjected into nuclei of XPA-deficient human fibroblasts. One day after

microinjection a bright fluorescent signal within the nuclei of injected cells was observed (Fig. 1B). Fluorescent cells were recorded and assayed for their repair capacity by determining UV-induced UDS. As shown in Fig. 1C, the cells with green fluorescent nuclei (Fig. 1B) were also corrected (up to wild-type levels) for the severe UDS defect present in XP-A cells (noninjected neighboring cells). Both nuclear targeting and the complete restoration of UDS indicate that the His9-HA-eGFP tag does not interfere with the proper function of XPA when transiently expressed in XP-A cells.

Generation and characterization of cells expressing GFP-XPA

To investigate the in vivo distribution of GFP-XPA in time and space, the fusion gene was stably expressed in an XPA-deficient human SV40-immortalized fibroblast. We isolated several UV-resistant clones and analyzed the expression levels of the fusion proteins by immunoblotting (data not shown). We selected a clone (clone 40) that expressed the fusion protein to near normal levels compared with XPA expression in wild-type cells (Fig. 2). As observed previously, wild-type XPA migrates as two distinct bands in polyacrylamide gel electrophoresis (40 and 42 kDa) (17, 42, 49); the fusion protein is also present in two forms, migrating at the expected positions of ~68 and ~70 kDa. Immunostaining with anti-GFP (data not shown) revealed that there was no detectable free GFP present in the crude extracts. UV survival experiments demonstrated that tagged XPA restores the extreme UV-sensitive phenotype of XP-A cells to the wild-type range (Fig. 2B). This confirms the UDS results of the microinjection at physiological protein levels in stably expressing transformants.

GFP-XPA appeared to be homogeneously distributed in living nuclei (Fig. 2C and D), including the nucleoli. In approximately 40% of the cells, a few (1 to 5) bright fluorescent spots were observed. GFP signal in fixed cells (Fig. 2E) and immunofluorescence, anti-XPA (Fig. 2F) displayed a similar distribution as in living cells (Fig. 2D), except that nucleoli seemed devoid of XPA after immunofluorescence (Fig. 2F), similar to what has been reported previously (42). The lack of nucleolar staining by immunofluorescence might be caused by the fixation procedure which renders this highly condensed organelle less permeable for antibodies (63). Although UV-C irradiation slightly reduced the number of cells containing spots, the overall distribution pattern did not change (data not shown). At present, the nature and significance of these foci remain unknown. Similar structures have been found in ERCC1-GFP-expressing cells (29).

Diffusion of GFP-tagged XPA

Prior to mobility studies of GFP-XPA in living cells, we first analyzed the expression level of individual cells in clone 40. Immunofluorescence with anti-XPA serum was performed on a (1:1) mixed population of wild-type (MRC5) and clone 40 cells. The frequency distribution (Fig. 2G) shows that both the level of expression and the intercellular variation are comparable, with the exception of a small fraction of cells overexpressing GFP-XPA. Only cells (from clone 40) with a modal expression level equivalent to that of wild-type cells were used in further quantitative fluorescent experiments, unless stated otherwise.

Mobility measurements to determine whether GFP-XPA molecules are mobile or bound to nuclear structures were performed by applying FRAP. Here we used the strip-FRAP method (see Materials and Methods and reference 30 for detailed information) to measure the mobility. Briefly, GFP-XPA molecules were photobleached in a defined narrow strip spanning the nucleus (strip-FRAP). The speed of recovery is a measure for the diffusion rate of the molecules, and the degree of fluorescence recovery indicates whether (part of) the GFP-XPA molecules are mobile.

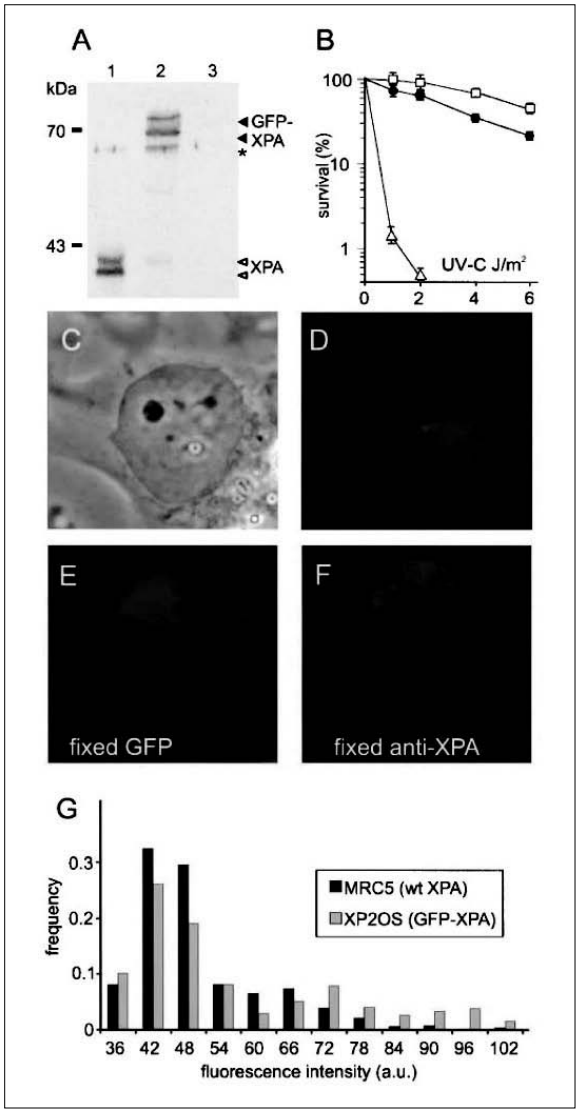

Figure 2. Expression and characterization of XP2OS cells stably expressing GFP-tagged XPA
(A) Immunoblot of 30 µg of whole-cell extract from MRC5, wild-type (lane 1), GFP-XPA-transfected XP2OS (clone 40) (lane 2), and XP2OS, XP-A (lane 3) cells probed with polyclonal anti-XPA. The molecular masses of protein markers are indicated in kilodaltons. No XPA protein was detected in XP2OS cells because of a G-C transversion in the splicing acceptor site in intron 3 of the XPA gene. The asterisk indicates a nonspecific crossreacting band. (B) UV survival of repair-proficient MRC5 cells (□), clone 40 (■) and XP2OS cells (△) (see Materials and Methods). The transfected cell line shows a wild-type correction of the XP-A-specific UV sensitivity. (C) Phase-contrast image of a living clone 40 cell. (D) Epifluorescence GFP image of the same cell as in panel C, showing a homogeneous nuclear distribution. (E) Fluorescence image after fixation of clone 40, showing a similar distribution as in panel D. (F) Immunofluorescence of the same cell as in panel E incubated with anti-XPA serum, showing a similar XPA distribution as with GFP fluorescence, except for the nucleoli. (G) Expression profiles of XPA (black bars) and GFP-XPA (gray bars) in MRC5 cells and clone 40, respectively, after immunofluorescence staining with XPA antibodies. GFP-XPA cells exhibiting an expression level similar to the major peak of XPA expression in MRC5 cells were used in further experiments. See the Appendix for a colour version of this figure.

For chemically fixed cells, no recovery was observed, as expected (Fig. 3A and B). In contrast, in living cells the vast majority of GFP-XPA appeared to be mobile (Fig. 3C and D). The kinetics of recovery yielded an effective diffusion coefficient (D_{eff}) for GFP-XPA of 15 ~2 µm²/s. This D_{eff} is much higher than that of XPB-GFP (6 ~1 µm²/s), part of TFIIH, and we found it in repeated experiments to be higher than that of ERCC1-GFP/XPF (12 ~2 µm²/s) assayed in parallel. These data suggest that in undamaged cells the majority, >95% of XPA is not incorporated into a stable large complex and diffuses freely as single molecules (or part of small transient subcomplexes) throughout the nucleoplasm.

DNA repair-dependent immobilization of GFP-XPA

To investigate the effect of the presence of DNA damage on XPA mobility, we performed FRAP analysis on cells exposed to UV-C (16 J/m^2, an NER-saturating dose) (Fig. 3E and F). The reduced fluorescence recovery, visible 2 s after bleaching (Fig. 3F [compare with nontreated cells shown in Fig. 3D]), and the lower recovery of the diffusion plot (Fig. 3G) are indicative for an immobilized fraction. The relative amount of binding (maximally ~35%) depends on the expression level, since cells expressing high levels of GFP-XPA show a proportionally smaller immobilized fraction compared with cells expressing moderate amounts of GFP-XPA (Fig. 3G). This suggests that at a given UV dose the total number of molecules participating in the DNA repair reaction (i.e., immobilization) is roughly the same in all cells independent of the expression level. The D_{eff} of the free fraction of GFP-XPA molecules did not change after damage induction (13 ~2 μm^2/s) (Fig. 3G), indicating that the free molecules were not incorporated into larger (mobile) complexes.

To more precisely quantify the DNA damage-induced immobilization of GFP-XPA, we used a different FRAP procedure, FRAP-FIM, as described previously (29, 30). FRAP-FIM measurements (the mean results for at least 30 cells and typical examples of cells) are shown in Fig. 4. In accordance with the strip-FRAP analysis, these measurements showed a UV light-dependent (and maximally ~35%) immobilization of GFP-XPA (Fig. 4G to J). These results indicate that the number of immobilized molecules depends on the number of lesions. In addition, the total amount of immobilized molecules, as shown above, does not depend on the amount of available GFP-XPA molecules, suggesting that XPA is not the rate-determining factor of NER in the cell line investigated. Note that these experiments have been performed using SV40-im-mortalized cells that have reduced levels of tp53, which causes a decrease in GGR capacity (8).

The fraction of bound GFP-XPA molecules (~35%) remained more or less unaltered over a period of a few (2 to 4) hours post-UV exposure. Subsequently, a gradual decrease of immobilized molecules was observed, with no significant binding 24 h after UV exposure (data not shown). Previous studies ported for ERCC1-GFP (29), and not observed in cells ex-have shown that 24 h after UV exposure most UV-induced pressing single GFP or GFP-tagged RAD52 group proteins lesions have been removed by NER, suggesting full release of (19). In summary, the above findings are consistent with the bound molecules when repair is completed. This UV-induced idea that after DNA damage GFP-XPA molecules become immobilization is specific for NER proteins, as previously re-transiently immobilized by engagement in NER.

Local damage in GFP-XPA cells

To monitor the translocation of GFP-XPA molecules to damaged DNA in living cells and to determine the transient binding (or residence) time within NER complexes, we applied a novel technique for introducing UV damage to a restricted area of the nucleus (43, 60). Cells were covered with a polycarbonate filter that shields UV light and contains (5 μm-diameter) pores (Fig. 5A), causing DNA damage after UV exposure only at the positions of the pores. Shortly after UV irradiation (<5 min, i.e., the first time point analyzed), a clear accumulation of GFP-XPA molecules in restricted parts of the nuclei of living cells was observed (Fig. 5C, arrows). These GFP-XPA accumulations colocalize with XPC (Fig. 5D and E) and with CPDs (see Fig. 7D and E) and confirm that XPA preferentially localizes to sites of DNA damage (60). These observations suggest a model in which, free diffusing GFP-XPA molecules bind rapidly to damaged DNA-NER complexes in which they were transiently entrapped. To determine the residence time of GFP-XPA within these locally damaged areas, we applied FLIP (see Materials and Methods). At a position opposite to the damaged domain, a single pulse was used to bleach a small region

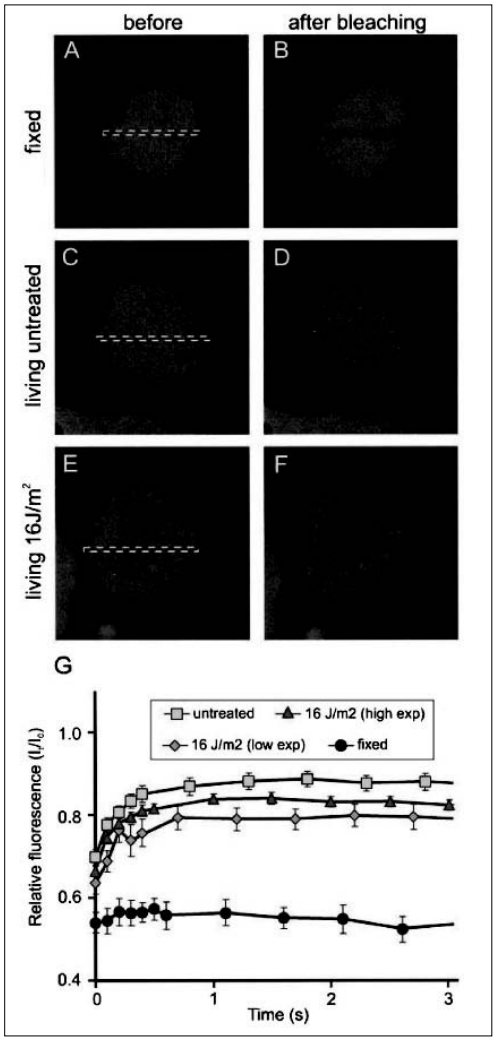

Figure 3. Temporal FRAP analysis applied on GFP-XPA-expressing cells to determine the immobile fraction after UV irradiation
Shown are confocal images and corresponding FRAP profiles. The dotted line indicates the position of the photobleaching strip. To determine a potential immobile fraction, the mean intensity immediately before bleaching was set as 1 and the fluorescence intensity immediately after bleaching was set as 0. (A and B) Pre- and postbleach images, respectively, of fixed cells, showing complete immobilization of GFP-XPA.
(C and D) Images of living cells monitored during FRAP, with panel D showing a homogeneous bleaching throughout the nucleus 4 s after the bleach pulse. (E and F) Images of living cells irradiated with 8-J/m^2 UV-C. Note that the UV-irradiated cell (F) shows an intermediate pattern between those of untreated (D) and fixed (B) cells. (G) Fluorescence recovery profile expressed as relative fluorescence plotted against time after bleaching. Each plot is the mean value for at least 50 cells, fixed cells, untreated living cells, UV-irradiated cells expressing relatively low levels of GFP-XPA, and UV-irradiated cells expressing high levels of GFP-XPA. The immobile fraction can be calculated by measuring the reduction of fluorescence recovery compared with nonirradiated cells.

in the nucleus (Fig. 6B, inset). Both bleached and nonbleached molecules will distribute and mix, resulting in an overall decrease in fluorescence intensity. The time required to establish the initial (prebleach) fluorescence difference between the damaged area and the nucleoplasm is a measure for the mean residence time of molecules in that area. A typical series of images of this FLIP measurement is shown in Fig. 6A. The residence time for GFP-XPA was determined to be approximately 4 to 6 min (Fig. 6B). This residence time indicates the average binding period, or time of participation of GFP-XPA molecules within a single NER event.

GFP-XPA mobility in an XPC-deficient background

To obtain further evidence that immobilization is caused by actual engagement in NER, we studied the dynamic properties of GFP-XPA in a mutant NER background, i.e., an XPC-deficient (XP20MA-SV) cell line. Using fluorescence-activated cell sorting and immunoblot analyses (data

not shown), a clone was selected that expresses a relatively low level of GFP-XPA, approximately a 1:1 ratio with endogenous nontagged XPA.

FRAP-FIM measurements revealed that even after a high UV dose of 16 J/m^2 no significant immobilization of GFP-XPA was observed in XP-C cells (Fig. 7A and B). To study the absence of immobilization in an XPC-deficient background in more detail, local damage was applied to GFP-XPA-expressing, XPA- and XPC-deficient cells. GFP-XPA accumulates at locally damaged areas (using CPD antibodies) (Fig. 7C to E) only in the presence of XPC (Fig. 7, compare panels C to E with panels F to H [absence of XPC]). These observations clearly show that binding and transient immobilization of XPA molecules to damaged regions depend on the presence of functional XPC. Since XPC is only involved in GGR, these results further suggest that with the applied FRAP methods (and GFP-XPA), predominantly GGR is monitored, at least in the analyzed time periods after UV exposure.

Loading of XPA and RPA in the NER preincision complex

Our experiments indicated that incorporation of XPA into the NER preincision complex depends on the presence of XPC and that the XPA molecules get access to these lesions as a free diffusing entity. These dynamic studies do not, however, allow the determination of whether (part of the XPA) molecules are complexed to other (small) nuclear factors, such as RPA. It has been suggested that XPA is bound to the heterotrimeric RPA (26, 37, 39). Moreover, it has

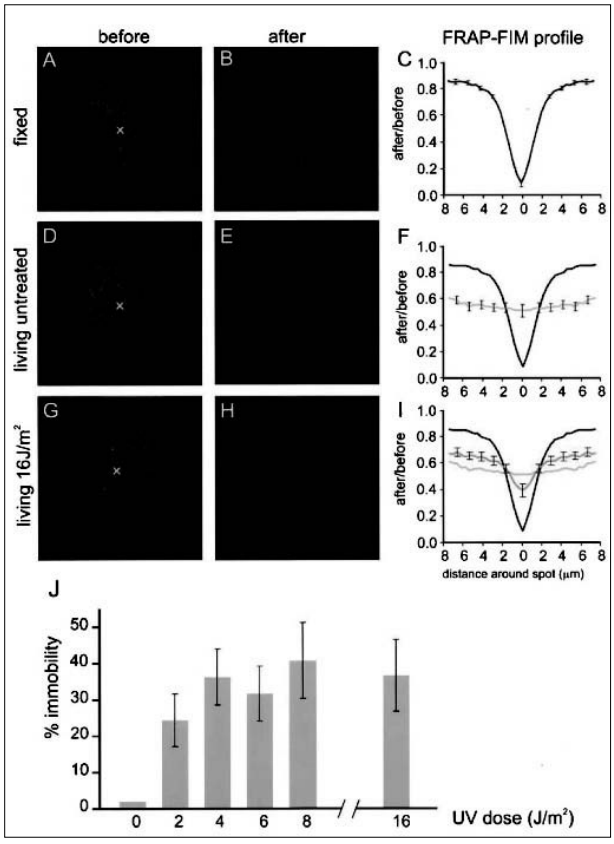

Figure 4. FRAP-FIM method applied to GFP-XPA-expressing cells
Shown are confocal images and corresponding fluorescence ratio profiles (FRP) of 50 cells. (A and B) Pre- and postbleach images, respectively, of cells fixed with 2% paraformaldehyde, displaying the immobilization of GFP-XPA molecules after fixation, visualized by the intense bleached spot and high fluorescence intensity outside the bleached spot, (B). (C) FRP of fixed cells. (D and E) Images of living untreated cells, showing an overall reduction of fluorescence after the bleach pulse (E). (F) FRP of untreated cells (green line). (G and H) Image of cells irradiated with 8-J/m^2 UV-C. The UV-irradiated cell (H) displays a distribution pattern intermediate of those of untreated (B) and fixed (E) cells. The 'X' in panels A, D, and G represents the position of the bleach pulse. (I) FRP of UV-irradiated cells (blue line). (J) Response of GFP-XPA immobilization to different UV doses.

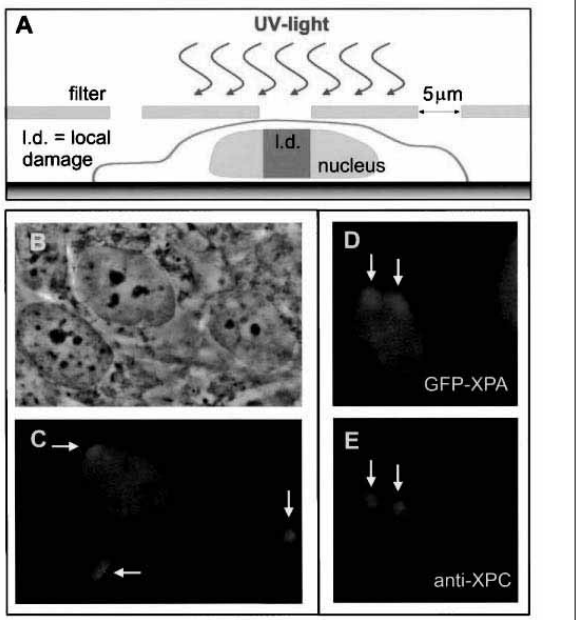

Figure 5. Accumulation of GFP-XPA within restricted nuclear areas after local UV irradiation (A) Schematic presentation of local UV damage infliction on living cultured cells. (B and C) Micrographs, phase-contrast image [B] and fluorescence image [C] of living cells expressing GFP-XPA (clone 40) and UV-irradiated through a filter with small (5 µm-diameter) pores. The arrows in panel C point to the local accumulations of GFP-XPA. (D and E) GFP-XPA accumulations (arrows) shown in panel D clearly colocalize with endogenous XPC (E) concentrations, as determined with anti-XPC antibodies, in fixed cells. See the Appendix for a colour version of this figure.

been claimed that these proteins bind as a complex to DNA damage (61, 62). This XPA-RPA complex has a higher and more lesion-discriminative binding capacity than each of the separate proteins (41). To further investigate whether XPA gets access to the preincision complex as a single protein or in conjunction with RPA, we investigated the loading of RPA on UV lesions in a number of different NER factor-deficient cells.

In agreement with previous findings for other NER proteins (60), we found an accumulation of RPA in normal cells shortly after the introduction of local UV damage in the cell (Fig. 8A to C). The recruitment of XPA to the NER complex is not impaired in XP-G/CS cells (60), despite the virtual absence of XPG protein. Here we show that the relocalization of RPA to locally induced UV damage is also not impaired in these cells (Fig. 8D to F). This observation suggests that the recruitment of XPA and RPA is independent of XPG or alternatively that the XP-G/CS cells investigated might still express small amounts of truncated XPG protein that are sufficient to support recruiting of XPA and RPA.

Surprisingly, however, when we tested cells lacking XPA we also observed relocalization of RPA to the UV-damaged area of the nucleus with an efficiency similar to that in wild-type cells (Fig. 8G to I). This suggests that RPA is recruited to the NER complex on the basis of its affinity for single-stranded DNA, which is formed after the helix-unwinding action of TFIIH, or that other (protein-protein) interactions than those with XPA are sufficient to recruit RPA to the NER complex. Support for the latter explanation comes from our observations with XP-B/CS cells, which have been reported to lack helix opening in NER (20). In these cells, we could still observe RPA accumulation in sites of local UV damage (Fig. 8J to L). The same result was obtained with an XP-D/CS cell line bearing a mutation in the other helicase subunit, XPD of TFIIH (data not shown).

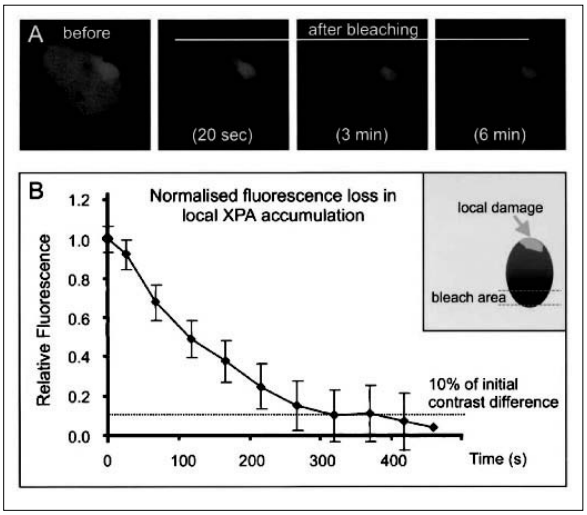

Figure 6. Application of FLIP, using locally damaged cells, to determine the binding time of GFP-XPA molecules on damaged DNA
(A) Confocal images of a locally UV-damaged GFP-XPA-expressing cell (clone 40). Shown are images before application of a bleach pulse and at 20 s, 3 min, and 6 min after bleaching. (B) Fluorescence profile as a function of time; residence time was estimated to be ~4 to 6 min, the time at which the relative fluorescence difference between damaged area and background is established to be <10% of the initial (prebleach) situation. The inset shows the application of the FLIP procedure in local damaged cells: a laser beam is focused (between dotted lines opposite to the damaged area (arrow) (a typical example is shown in panel A).

These findings are in accordance with recent observations by Patrick and Turchi (47), who presented evidence that initial binding of RPA to the damaged DNA is subsequently further stabilized by an interaction with XPA. Whether XPA is incorporated in the NER complex in vivo in the absence of RPA remains to be determined.

Furthermore, it has been suggested by Wakasugi and coworkers that the DDB protein complex might be responsible for the direct recruitment of XPA and RPA to sites of damage, without the need for the XPC/hHR23B complex (62). However, when we investigated XPC-deficient cells (that contain normal functional DDB complex) we found no accumulation of RPA in sites of local UV damage (Fig. 8M and N), in accordance with our previous findings that no NER proteins, including XPA, were found at sites of locally induced UV damage in XP-C cells. This observation together with our group's present and previous observations (60) support the notion that the visible assembly of the NER complex on a DNA lesion strictly depends on functional XPC protein, and we have found no evidence for direct recruitment of XPA and/or RPA by the DDB protein. Importantly, our observations are in contrast with the new order of assembly recently suggested by Reardon and Sancar (48), who describe a model in which RPA loading precedes XPC.

Discussion

The XPA protein plays an essential role in mammalian NER that until now has largely been assessed using biochemical and genetic means. In this study, we applied different variants of FRAP (30) on living cells stably expressing (at a biologically relevant level) functional GFP-XPA. These analyses provide insight into the hitherto unexplored in vivo spatiotemporal organization of this central NER factor.

The following observations indicate that the GFP-XPA-expressing cells accurately reflect the in vivo involvement of XPA in NER. (i) Expression in XPA-deficient cells established that the GFP-XPA protein is fully functional in NER in terms of UV survival and repair synthesis, despite the presence of the GFP tag, almost doubling the size of the protein. (ii) GFP-XPA is expressed at physiological levels, which is critical when analyzing the biologically active fraction of the

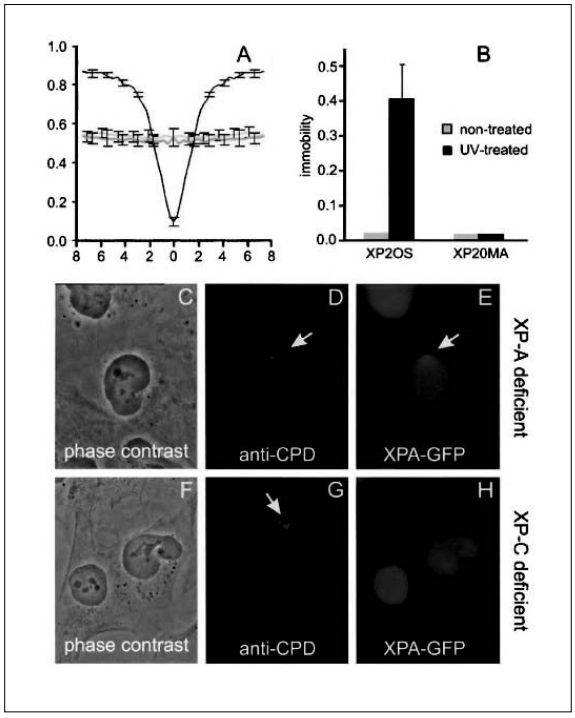

Figure 7. Effect of XPC on damage-induced XPA immobility as analyzed by FRAP-FIM and local damage induction
(A) FRAP-FIM profile of GFP-XPA expression in XP20MA (XP-C) cells. Shown are results for nonirradiated cells (light green line), cells irradiated at 16 J/m^2 (blue line), and fixed cells (black line). UV-exposed XP20MA cells do not show any GFP-XPA immobilization. (B) Quantification of immobilization of GFP-XPA in XP20MA and XP2OS cells with and without UV irradiation. (C) Phase-contrast image of GFP-XPA-expressing XP2OS cells. (D) Anti-CPD immunostaining in a GFP-XPA-expressing XP2OS cell. The arrow indicates the site of the damage. (E) GFP image of the same cell as in panel D, showing enrichment of GFP-XPA at the damaged site. (F) Phase-contrast image of GFP-XPA-expressing XP20MA cells. (G) Anti-CPD immunostaining of a GFP-XPA-expressing XP20MA cell, indicated by the arrow. (H) GFP image showing no enrichment of GFP-XPA molecules. See the Appendix for a colour version of this figure.

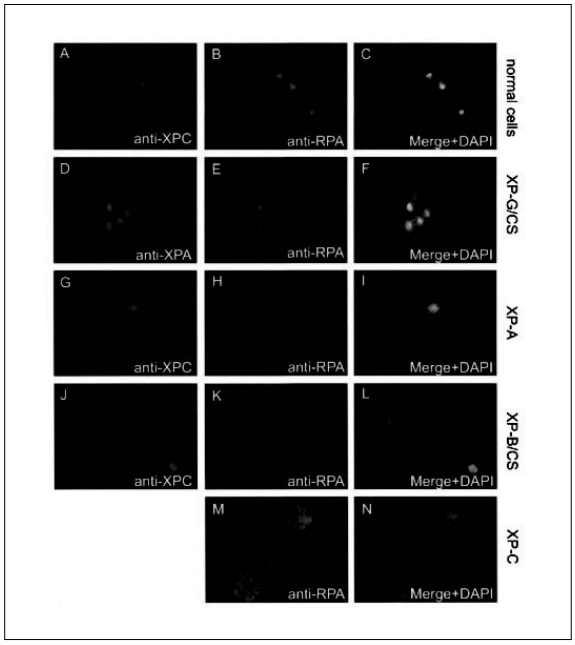

Figure 8. Relocalization of RPA to the NER complex 30 min after local irradiation with 25 J/m^2 UV
(A to C) Anti-XPC (A) and anti-RPA (B) immunostaining of VH25 cells and the merged image after immunostainings plus DAPI nuclear DNA staining (C). (D to F) Anti-XPA (D) and anti-RPA (E) immunostaining of XPCS1RO cells and the merged image after immunostainings plus DAPI nuclear DNA staining (F). (G to I) Anti-XPC (G) and anti-RPA, H immunostaining of XP25RO cells and the merged image after immunostainings plus DAPI nuclear DNA staining. (J to L) Anti-XPC (J) and anti-RPA (K) staining of XP131MA cells and the merged image after immunostainings plus DAPI nuclear DNA staining (L). (M and N) Anti-RPA immunostaining of XP21RO cells (M) and the merged image after immunostaining plus DAPI nuclear DNA staining (N). The yellow color in the merged images in panels C, F, I, and L indicates colocalization of NER proteins at sites of locally induced DNA damages. See the Appendix for a colour version of this figure.

protein. (iii) The subnuclear distribution is similar to that of wild-type, endogenous XPA (Fig. 1 and 2). (iv) As discussed below, the protein shows a consistent, direct, and specific response to NER-type DNA injury. Therefore, we consider this cell line to be a bona fide tool for studying the characteristics of XPA in living cells.

Organization of NER in living cells

Diffusion measurements in living cells indicated that the majority of GFP-XPA molecules are not part of a large, stable, preassembled NER complex. Obviously, we cannot exclude the possibility that a small fraction of XPA is incorporated into a larger functional NER (holo)complex. However, it is hard to envisage that such a small amount would be sufficient to account for the observed biological activity that involves a substantial fraction (>30%) of the XPA proteins actually participating in NER (Fig. 4). The absence of (significant quantities of) preassembled NER holocomplexes containing XPA (as determined here) in living cells contrasts with the results of an earlier biochemical study of *Saccharomyces cerevisiae* in which a completely assembled NER complex was identified (54). It is, however, not excluded that part of the XPA molecules are present in smaller, transient complexes, as reported previously (3, 16, 23, 61). Several explanations may account for these differences. First, during cell lysis and extract preparation, ionic-strength and local concentrations are different from those in the in vivo situation. In addition, competing factors, or natural substrates, such as DNA and/or chromatin, are absent. This will affect (and may even enhance) associations between proteins with an intrinsic affinity for each other, influencing copurification and immunoprecipitation behavior. Second, many reported interactions between NER factors are based on two-hybrid screens or immobilized factors on column matrices (reviewed in references 5 and 15). In both of these cases, high local concentrations of one NER factor will artificially shift the association-dissocia-tion equilibrium to the side of binding. Finally, the process of an ordered repair complex assembly in mammalian nuclei (60) might differ from the situation in yeast cells due to differences in genome size and nuclear structure.

Transient immobilization of GFP-XPA in DNA repair

Here we provide evidence that GFP-XPA immobilization is linked to actual repair and involves sequential NER complex assembly on DNA lesions in living cells. Immobilization of GFP-XPA is likely due to either direct sequestration to damaged DNA or entrapment into NER reaction intermediates. First, free diffusing GFP-XPA became partially immobilized when DNA damage was inflicted by UV light. Our in vivo results are consistent with the previously reported binding of XPA to nuclear structures after UV irradiation as determined by reduced Triton X-100 extractability on fixed cells (55). Second, the UV-induced immobilization is found specifically for NER factors and is not noted with other proteins, such as transcription activators and proteins implicated in other repair pathways (19, 29). Third, the fraction of immobilized GFP-XPA appears to depend on the number of lesions induced by UV irradiation. Fourth, we observed a time-dependent reduction of the amount of trapped GFP-XPA after UV irradiation, with no significant binding 24 h post-UV exposure. This is in agreement with the notion that when DNA repair proceeds, fewer target sites are available for binding GFP-XPA. Fifth, as discussed below, XPA immobilization does not occur in an XPC-deficient background. Finally, we visualized a fast recruitment of GFP-XPA to areas of local UV damage in nuclei of living cells. FLIP measurements indicated that GFP-XPA molecules reside for approximately 4 to 6 min within these locally damaged sites.

The transient UV dose-dependent immobilization of GFP-tagged ERCC1 in CHO cells and that of GFP-XPA analyzed here in human cells show comparable kinetics in terms of the maximum fraction of molecules that become immobilized and the UV dose at which both reach a plateau. The rate at which both proteins accumulate in damaged regions of nuclei (data not shown) and their residence time at these areas are quite similar as well. The observed comparable reaction kinetics, on rate, binding time, and substrate (UV damage) dependency suggest that both factors enter the NER complex, stay bound, and are subsequently released from the DNA lesion-NER complex at about the same time. A marked difference, however, is that in the CHO cell line expressing ERCC1-GFP the total repair time (i.e., time after UV irradiation where no notable immobilization of ERCC1-GFP is observed anymore) is significantly shorter than in the case of GFP-XPA. This difference is likely due to the virtual absence of CPD removal of nontranscribed DNA (GGR) in rodent cells, in contrast to the more complete repair of CPDs in human cells (31).

Order of NER factor assembly

Both UV-dependent immobilization (Fig. 7A and B) and accumulation of GFP-XPA at nuclei with local UV damage depend on the presence of XPC (Fig. 7C to H). These findings provide in vivo evidence supporting the results of previous biochemical studies indicating that the action of XPC/hHR23B precedes the XPA involvement in NER (6, 52, 53, 64). They are also in line with previous immunocytochemistry analysis results (60) indicating that assembly of various NER factors (XPA, TFIIH, ERCC1/XPF, and XPG) at a local damaged area is dependent on XPC.

The major, and perhaps only difference between TCR and GGR is based on the (initial) recognition step. It is therefore also likely that the factors that are different in the two pathways, i.e., XPC/hHR23B (and for some lesions UV-DDB [or XPE]) (56) and the CS factors, respectively, for GGR and TCR, are the respective damage sensors for both NER subpathways. In addition, XP group A cells are deficient in both GGR and TCR, whereas XP-C and CS cells are selectively defective in GGR and TCR, respectively, which argues against an initial damage-sensing role for XPA. Since XPC only accounts for GGR, it is surprising that in XP-C cells, no local accumulation of GFP-XPA was observed, whereas these cells have a normal functional TCR. Apparently, the contribution of TCR in these cells is too low to be detected by the currently applied immunocytochemistry procedure using local irradiation or FRAP-FIM techniques.

Surprisingly, we also found that RPA is localized to UV-damaged subnuclear regions in the absence of XPA, suggesting that at least for the loading of RPA into the NER preincision complex, association to XPA is not required. In addition to the observed diffusion rates of GFP-XPA, it is therefore conceivable that the majority of XPA and RPA are not complexed prior to binding to the NER lesion. In view of the multiple roles of RPA, it is also plausible that association of RPA to its interacting partners, here XPA, only occurs after entrance to the specific site of action.

Furthermore, local accumulation of RPA to UV-damaged areas seemed to depend on functional XPC, whereas XPA and XPG appeared to be dispensable for this relocalization. However, it cannot be excluded that the severely truncated XPG protein present in XP-G/CS cells is sufficient to (make it possible to) recruit RPA to the NER complex. A further point of interest is the observation that the functionality of the helicase subunits of TFIIH (XPB and XPD), which cause the formation of a single-stranded DNA region around the lesion that could theoretically attract RPA to the site of NER, is in fact dispensable for this recruitment. Our results therefore indicate that RPA is also not incorporated into the forming NER complex on the basis of its

affinity for this single-stranded DNA region only but may require protein-protein interactions of early NER factors. These findings raise the question as to what causes the entry of RPA into the NER complex, i.e., which protein(s) or protein function(s) is necessary and indispensable for RPA to be recruited to the NER complex, and the timing of this entry. Theoretically, given our findings, it is possible that RPA enters the NER complex as the third protein (complex), after XPC/hHR23B/centrin 2 and TFIIH, in order to be incorporated into the NER incision complex. At present, however, we cannot exclude the possibility that the XPG protein precedes this step.

Advantages of a sequential assembly model for NER

This work combined with our group's previous study (29) shows that repair factors XPA and ERCC1/XPF participate in NER by a temporary entrapment of free diffusing proteins into NER-DNA lesion complexes. These results favor an 'assembly on the spot' model for individual NER factors rather than a model of preassembled NER complexes. Preassembled 'repairosomes' might be considered to be efficient 'machines' ready to act on demand. On the other hand, dynamic assembly and disassembly of molecular complexes allows a more combinatorial flexibility of the reaction constituents that participate in other mechanisms. This is particularly relevant for NER, since almost all NER factors, except XPA, are known to participate also in other DNA-metabolizing processes. TFIIH and CSB are involved in transcription (50, 58), ERCC1/XPF functions also in recombination repair (46), XPG plays an additional role in base excision repair, BER (12, 36), and the single-stranded DNA binding protein hRPA acts in almost every DNA transaction. The latter is perhaps the prototype of a multilateral factor, since this hRPA functions in at least replication, NER, BER, and homologous recombination (34, 40). The different repair proteins (from BER, NER, and doublestrand break repair) interact with a common small domain of this protein, arguing for a competitive association with RPA, rather than divers preassembled subcomplexes including RPA specific for each cellular function. A distributive, diffusion-driven model has the advantage of permitting efficient usage and quick switching of proteins or protein complexes between distinct nuclear processes. An additional important advantage of sequential assembly of NER factors is that it allows regulation at multiple levels.

The involvement of many NER factors in other processes may imply that the basic rules learned here for NER are also applicable to those other systems, at least when shared NER factors are concerned. Nuclear processes, such as replication and transcription, are scheduled and confined or initiated at specific loci in the genome. These mechanisms may require structural nuclear elements that coordinate their specific spatial and temporal actions. High local concentrations of specific factors have indeed been found, and models have been put forward in which DNA is pulled through these 'factories' (11). However, such a spatiotemporal regulation might be less beneficial for repair than for replication and transcription, since repair has to act at any location in the genome at any moment in the cell cycle. Free diffusion of repair factors and binding when affinity is increased by a structural change (lesion) seems to be more efficient than tracking along relatively crowded DNA stretches or chromatin fibers by large complexes over long distances before they encounter injuries. However, a partial scanning mode of action is not excluded for all NER factors. Lesion detection within TCR is by definition performed by a DNA-tracking RNA polymerase II elongation complex. A similar scenario for the initial step in GGR can be envisaged.

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Chapter 9

The UV-damaged DNA binding protein mediates efficient targeting of the nucleotide excision repair complex to UV-induced photolesions

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9 The UV-damaged DNA binding protein mediates efficient targeting of the nucleotide excision repair complex to UV-induced photolesions

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Abstract

Previous studies point to the XPC-hHR23B complex as the principal initiator of global genome nucleotide excision repair (NER) pathway, responsible for the repair of UV-induced cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) in human cells. However, the UV-damaged DNA binding protein (UV-DDB) has also been proposed as a damage recognition factor involved in repair of UV-photoproducts, especially CPD. Here, we show in human XP-E cells (UV-DDB deficient) that the incision complex formation at UV-induced lesions was severely diminished in locally damaged nuclear spots. Repair kinetics of CPD and 6-4PP in locally and globally UV-irradiated normal human and XP-E cells demonstrate that UV-DDB can mediate efficient targeting of XPC-hHR23B and other NER factors to 6-4PP. The data is consistent with a mechanism in which UV-DDB forms a stable complex when bound to a 6-4PP, allowing subsequent repair proteins – starting with XPC-hHR23B – to accumulate, and verify the lesion, resulting in efficient 6-4PP repair. These findings suggest that (i) UV-DDB accelerates repair of 6-4PP, and at later time points also CPD, (ii) the fraction of 6-4PP that can be bound by UV-DDB is limited due to its low cellular quantity and fast UV-dependent degradation, and (iii) in the absence of UV-DDB a slow XPC-hHR23B-dependent pathway is capable to repair 6-4PP, and to some extent also CPD.

Keywords: UV-damaged DNA binding protein; Xeroderma pigmentosum group E; 6-4 Photoproducts; Cylcobutane pyrimidine dimmers; Nucleotide excision repair

1 Introduction

The nucleotide excision repair (NER) system is a highly versatile repair pathway capable of removing a wide variety of helix-distorting lesions from genomic DNA. Cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP), the main products of short-wave ultraviolet (UV) radiation in DNA, are among the lesions processed by NER, and human cells depend solely on NER to repair these UV-photolesions. Defects in genes encoding NER proteins give rise to UV-sensitive disorders, most notably xeroderma pigmentosum (XP), which comprises seven complementation groups (XP-A–XP-G) (1). XP patients are photosensitive and display a strongly elevated risk of developing skin cancers in sunlight-exposed parts of the body.

Biochemically, all XP patients (except XP variants) are found to be defective in global genome repair (GGR), the subpathway of NER capable of removing lesions from the entire genome. In

GGR, a total of ~30 proteins work together to consecutively execute the basic steps that comprise NER: recognition of the DNA damage, unwinding the DNA around the lesion, excision of the oligonucleotide containing the damage and finally resynthesis of the strand using the undamaged template and ligation of the nick (2). Several specialized proteins have been proposed to act in the initial step of damage recognition, including the UV-damaged DNA binding protein (UV-DDB) (3,4), the complex of XPA and replication protein A (RPA) (5,6) and the XPC-hHR23B heterodimer (7). Most recent studies point to the XPC-hHR23B complex as the principle initiator of NER (8,9). However, those studies did not exclude the possibility that in processing certain lesions, e.g. UV-induced photoproducts, the action of XPC-hHR23B in the formation of the incision complex can be preceded by other proteins such as UV-DDB.

UV-DDB is a heterodimer of the p48 and p127 proteins (10), products of the *DDB2* and *DDB1* genes, respectively. Microinjection of purified UV-DDB was originally found to restore the repair defect of XP-E cells as measured by unscheduled DNA synthesis (11). Recently, evidence has accumulated implying that the XP group E phenotype is caused exclusively by mutations in the *DDB2* gene, implicating that the *DDB2* gene corresponds to the *XPE* gene, and p48 to the XPE protein (12–14).

In vitro binding studies have revealed that the UV-DDB protein complex exhibits a high affinity for UV-induced DNA lesions and a moderate affinity for several other types of DNA lesions (3,15–17). Moreover, UV-DDB has an equally high specificity for 6-4PP when compared to the XPC protein, but a much higher affinity for DNA in general (3,16). A high affinity for DNA damage would be consistent with a role for UV-DDB in the recognition of these lesions directly after their infliction. Results of in vitro repair experiments to assess a role of UV-DDB in NER are inconclusive. Extracts from XP-E cells lacking UV-DDB did not show reduced repair of UV-irradiated DNA nor of an oligonucleotide substrate harbouring a single cisplatin lesion when compared to normal human cells (18). Also, the addition of UV-DDB to a reconstituted system stimulated repair only moderately, and even led to inhibition of the reaction at high concentrations (19). In contrast to the abovementioned studies, a recent study by Wakasugi et al. revealed that in their reconstituted repair system using purified factors, the repair efficiency of CPD and 6-4PP was enhanced by UV-DDB, particularly in the case of CPD (20). This result implies that the role of UV-DDB is not limited to repair inside a chromatin context but instead UV-DDB can stimulate repair through direct interaction with the DNA lesion.

Several studies reported that efficient global genome repair of UV damage in vivo did require UV-DDB (4,11,21), suggesting that the protein might be involved in the repair of lesions within a chromatin context. Indeed, p48 shares homology with chromatin reorganising proteins (22) and UV-DDB interacts with the CBP/p300 histone acetyl transferase (23,24), consistent with a function in remodelling of chromatin to allow efficient repair in the vicinity of the lesion.

In vivo studies have shown that p48 and p127 relocalise rapidly to sites containing UV-damaged DNA immediately after irradiation even in the absence of functional XPC protein (20,25). This observation is consistent with a role for UV-DDB in UV damage recognition, even before XPC is involved. However, the impact of the rapid recruitment of UV-DDB to UV damages on the efficiency of NER remains unclear.

Rodent cells efficiently repair 6-4PP, but in marked contrast to humans, show almost no repair of CPD in the genome overall. This marked difference in CPD repair phenotype between human and rodent cells has been attributed to the absence of UV-DDB activity in rodent cells (21). The situation in rodent cells has led to the well-established notion that UV-DDB is essential for repair of CPD by GGR. However, since the affinity of UV-DDB for 6-4PP is considerable higher than

that for CPD (16), UV-DDB might contribute also to GGR of 6-4PP in human cells, the rate of which greatly exceeds that of CPD. Indeed, an XP-E patient with defective repair of 6-4PP was reported (26). In contrast, in other reports repair of 6-4PP in the absence of UV-DDB was either not affected or only delayed (4,21), indicating that XPC-hHR23B by itself is capable of efficiently recognizing and targeting 6-4PP for repair.

In this study, we assessed the NER incision complex formation and repair of 6-4PP in local spots of UV damage, in cells proficient or deficient for UV-DDB. We find that both the accumulation of NER complexes and 6-4PP repair are greatly enhanced in the presence of UV-DDB. Moreover, we present evidence that the cellular quantity of UV-DDB is the limiting factor for this enhanced 6-4PP repair. We propose a mechanism in which UV-DDB accelerates GGR of 6-4PP by binding to the lesion, facilitating the recruitment of XPC and subsequent factors. In the absence of UV-DDB, 6-4PP are repaired by the GGR pathway dependent solely on recognition by XPC-hHR23B, leading to slower but nevertheless complete repair of 6-4PP.

2 Materials and methods

2.1 Cell culture

Primary diploid human fibroblasts used for immunofluorescence studies, derived from a normal individual (VH25) and xeroderma pigmentosum group E patients (XP2RO and XP23PV), were grown in Ham's F10 medium from which hypoxanthie and thymidine were omitted, and supplemented with 15% fetal calf serum and antibiotics at 37°C in a 2.5% CO_2 atmosphere. XP23PV cells were kindly provided by Dr. M. Stefanini (Instituto di Genetica Molecolare, Pavia, Italy). Simian virus 40 (SV40)-immortalized human fibroblasts, wildtype (MRC5) and XP-A (XP12RO), and wildtype Chinese hamster cells (V79B), were cultured under similar conditions in a 5% CO_2 atmosphere. XP12RO cells stably expressing CPD-or 6-4PP photolyase (S. Nakajima and A. Yasui, manuscript in preparation) were kindly provided by Dr. A. Yasui (Nagoya University, Nagoya, Japan). We immortalized fibroblasts derived from a normal individual by telomerase transfection (VH10hTert)(27). Immortilized cells from a XP-E patient (GM01389hTert) were kindly provided by Dr. E.C. Friedberg (University of Texas, Dallas, TX). Both immortilized cells were grown in DMEM supplemented withh 10% fetal calf serum and antibiotics in a 5% CO_2 atmosphere. VH10hTert cells and XP12RO cells expressing photolyases were grown in the presence of 25 μg/ml and 0.5 mg/ml G418, respectively.

2.2 Construction of vectors expressing fusion proteins

Full-length murine *DDB1* cDNA was amplified by PCR using Hot Start GoldStar DNA polymerase (Eurogentec) with the primer set 5-CCG-GAA-TTC-C-GGA-GGC-ATG-TCG-TAC-AAC-TAC-GTC-GT-3′ (forward) and 5-TCC-CCG-CGG-CTA-ATG-GAT-CCG-AGT-TAG-CT-3′ (reverse) and digested with *Eco*RI (forward) and *Sac*II (reverse) endonucleases. The cDNA fragments were cloned in-frame into the *Eco*RI and *Sac*II site of pECFP-C1 (Clontech), resulting in a vector expressing a CFP-p127 fusion protein. Murine *DDB2* cDNA was amplified similarly, using the primers 5-CCG-GAA-TTC-CTC-TTC-ACC-GAG-TAC-GTC-AT-3′ (forward) and 5-CGC-GGA-TCC-CC-GCC-TCC-TAG-TCT-TTC-ATG-ATC-TTT-CT-3′ (reverse). To create a vector expressing p48-YFP fusion protein, after digestion of the cDNA with *Eco*RI and *Bam*HI, the cDNA fragments were cloned in-frame into the *Eco*RI and *Bam*HI site of pEYFPN1 (Clontech). A vector expressing a p48-FLAG fusion protein was created after PCR amplification of murine *DDB2* cDNA using the primer set 5-AT-CCC-AAG-CTT-CTC-TTC-ACC-GAG-TAC-GTC-AT-3′ (forward) and 5-AAG-GAA-AAA-AGC-GGC-CGC-GCT-GCC-ACT-CCT-CAC-AGA-AT-3′ (reverse) and digestion with *Hin*dIII and *Not*I. Resulting cDNA fragments were cloned in-frame into p3XFLAG-CMV (Sigma) at the *Hin*dIII and *Not*I sites to form the p48-FLAG vector, expressing the FLAG polypeptide fused to the C terminus of p48. All vectors contain a *NEO* gene, and a cytomegalovirus promoter to control expression of the fusion genes. After construction of the vectors, all fusion genes were sequenced to ensure that no mutations had been introduced during PCR and cloning.

2.3 Transient transfection of human cells

Immortalised XPA-deficient human fibroblasts stably expressing either CPD-or 6-4PP photolyases were subcultured 24 h before transfection, and subsequently grown in the *absence* of G418 to improve transfection efficiency. Cells were transfected with plasmids p48-YFP and CFP-p127 us-ing the FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. After transfection, cells were cultured for an additional 24 h to allow expression of the fusion proteins before experiments were carried out.

2.4 Global and local UV irradiation

Confluent cells were washed with phosphate-buffered saline (PBS), irradiated with a Philips TUV lamp (predominantly, 254 nm) at a dose rate of 0.2 W/m^2 as described previously (28). For local UV irradiation, cells on coverslips were washed once with PBS, covered with an isopore polycarbonate filter with pore sizes of 5 or 8 μm (Millipore, Bradford, MA) and UV-irradiated (29). Only half of a coverslip was covered with the filter for experiments with simultaneous local and global UV irradiation. After irradiation, the filter was removed, and cells were returned to culture conditions for times indicated. Photoproducts were removed from the genome of photolyase-expressing cells by exposing the cells for 1 h to light from a far-blue TL tube.

2.5 Antibodies

The following primary antibodies were employed: affinity-purified rabbit IgG polyclonals anti-XPC and antiERCC1, kindly provided by Hanny Odijk and Dr. Wim Vermeulen (Erasmus MC, Rotterdam, The Netherlands); affinity-purified mouse IgG monoclonal anti-RPA70, a gift from Dr. H.P. Nasheuer (National University of Ireland, Galway, Ireland); mouse IgG monoclonals anti-6-4PP and anti-CPD, gifts from Dr. O. Nikaido (Kanazawa University, Kanazawa, Japan); mouse IgG monoclonal anti-XPB, a gift from Dr. J.-M. Egly (IGMC, Illkirch, France); affinity-purified mouse monoclonals anti-XPA and anti-XPG, gifts from Dr. Rick Wood (UPCI, Pittsburg, PA). Secondary antibodies that were utilized are: FITC-conjugated donkey anti-mouse and donkey anti-rabbit IgG; Cy2conjugated goat anti-mouse IgG and goat anti-rabbit IgG; Cy3-conjugated goat anti-rabbit IgG and goat anti-mouse IgG + IgM (Jackson Laboratories, Westgrove, PA). Alexa Fluor 488-conjugated goat anti-mouse IgG was obtained from Molecular Probes (Leiden, The Netherlands). All secondary antibodies were used according to the manufacturer's instructions.

2.6 Fluorescent labelling

The fluorescent labelling was performed essentially as described (9). Briefly, cells were washed twice with cold PBS, fixed and lysed in PBS with 2% formaldehyde and 0.2% Triton X-100 for 15 min on ice and washed again twice with cold PBS. Cells were then incubated with 3% bovine albumin in PBS for 30 min at room temperature. To visualize CPD or 6-4PP, the cellular DNA was denatured with 0.1 M HCl for 10 min at 37°C. Primary and secondary antibodies were incubated for 2 h and 1 h, respectively, at room temperature in washing buffer (WB: PBS, 0.5% bovine albumin, 0.05% Tween-20). After each antibody incubation, cells were washed three times for 5 min with WB. Cells were mounted in Vectashield mounting medium containing DAPI (1.5 μg/ml) (Vector Laboratories, Burlingame, CA) or in Aqua/polymount (Polysciences Inc., Warrington, PA) containing DAPI (1.5 μg/ml) that gave identical results.

2.7 Microscopy and quantification of fluorescent signal

To capture fluorescence images, a Zeiss Axioplan 2 epifluorescence microscope fitted with appropriate filters coupled to an AttoArc HBO 100 W adjustable mercury arc lamp, and a Hamamatsu C5935 cooled CCD camera, were used. The pictures were captured and processed with Metasystems (Altlussheim, Germany) ISIS software. Using the ISIS software package, the total fluorescence intensity of the area to be quantified (whole nuclei or local UV spots) was measured and divided by the surface area, resulting in a specific fluorescence intensity expressed in arbitrary units.

2.8 Gene specific determination of 6-4PP

Cells were grown to confluency in petridishes to ensure that no cells were in S-phase during the experiments. Cells were UV-irradiated with 30 J/m^2, and subsequently incubated in culture medium, and lysed. High-molecular-weight DNA was isolated and purified as described (30). The DNA was restricted with

*Eco*RI, purified and CPD were removed from the DNA by in vitro photoreactivation employing the photolyase derived from *Anacystis nidulans*, kindly provided by Dr. A. Eker (Erasmus University, Rotterdam, The Netherlands) (30). Photoreactivation was checked for completeness by treatment of DNA samples with T4 endonuclease V and subsequent Southern analysis. Equal amounts of DNA (5 µg) were either treated or mock treated with UvrABC excinuclease (2 pmol of each subunit per µgDNA) or 228-UVDE (ultraviolet DNA endonuclease; (31)) endonuclease (5 pmol/µg DNA). UvrABC and 228-UVDE were kindly provided by M. de Ruiter (Leiden University, Leiden, The Netherlands). After incubation, 10 mM EDTA and 0.1% SDS were added and the DNA was purified by phenol and chloroform extraction, precipitated with ethanol and dissolved in TE. The samples were electrophoresed in 0.6% alkaline agarose gels. The DNA was transferred to Hybond N+ membranes by vacuum Southern blotting and hybridised with a ^{32}P-labeled gene-specific probe recognizing both strands of an 18.5 kb *Eco*RI fragment of the human ADA gene (exon 12). Filters were scanned using the Instant Imager (Packard Instrument Company). The number of 64PP per restriction fragment was calculated from the relative band densities of full size restriction fragments in the lanes containing DNA treated or not treated with either UvrABC excinuclease or UVDE endonuclease, using the Poisson expression.

The use of UVDE, a DNA repair enzyme of *Schizosaccharomyces pombe* that cuts at sites of DNA photolesions (31) was introduced in the latter assay to circumvent background cutting in DNA from mock-treated cells, a known property of UvrABC (30). Fig. 2 shows that UVDE does not exhibit non-specific cutting and hence, UVDE provides an important improvement over UvrABC.

3 Results

3.1 Repair of 6–4 photoproducts in normal human and XP-E cells after global UV irradiation

Repair of UV-induced cyclobutane pyrimidine dimers in human cells is dependent on functional p48 based on the results of biochemical and immunochemical analysis of photolesions in normal and XP-E cells (21). Data on repair of 6-4PP in XP-E cells are only available from immunoslotblot analysis and the results are less consistent than for CPD. Most studies failed to detect major differences in rates of 6-4PP repair between p48-proficient and -deficient cells after global irradiation (4,21), whereas a partial repair defect was reported by Itoh et al. (26). We examined the repair of 6-4PP in globally UV exposed normal human and XP-E cells by two different approaches: immunofluorescence and biochemical (gene specific) analysis. Gene specific analysis requires a relative high UV dose (30 J/m^2) to induce a single 6-4PP per gene fragment (30); hence, to allow comparison, both assays were performed with cells exposed to 30 J/m^2. In previous studies, we determined repair of 6-4PP at the gene level with purified UvrABC excinuclease complex to cut at sites of DNA damage (30). Since this report presents the first instance of UVDE endonuclease to replace UvrABC in the DNA cutting reaction, we validated the use of UVDE. First, the initial frequency of 6-4PP observed using UVDE is 0.0196 6-4PP per 10 kb fragment per J/m^2, which is virtually equal to the frequency found with UvrABC (30). Second, we find indistinguishable repair kinetics of 6-4PP in normal human cells when assayed by UVDE or UvrABC (Fig. 1). We assessed the kinetics of 6-4PP repair in normal human fibroblasts (VH25 and VH10hTert) and in human XP-E fibroblasts (XP23PV and GM01389hTert) exposed to 30 J/m^2 of UV light. Autoradiograms of representative experiments and graphs derived from scanning of these autoradiograms are shown in Fig. 2. In normal human cells, 6-4PP are removed fast from both strands of the *Eco*R1 fragment of the *ADA* gene: approximately 70–80% of the 6-4PP are repaired within 4 h and repair is virtually complete after 8 h. This is in agreement with results obtained in our previous studies (30). Furthermore, there is no difference in the kinetics of removal of 6-4PP between normal and

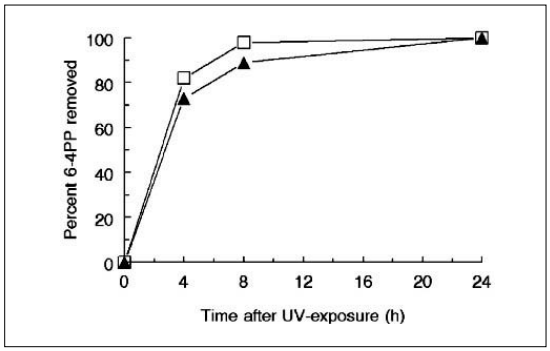

Figure 1. Similar kinetics of 6-4PP repair assayed by UvrABC or UVDE endonuclease
Normal human cells (VH25) were exposed to 30 J/m^2 of global UV. Removal of 6-4PP at various times after irradiation was measured in *Eco*RI restriction fragments of the active *ADA* gene (18.5 kb) using a probe recognising both strands. After photoreactivation of CPD, the DNA was cut using either (□) UvrABC excinuclease or (▲) UVDE endonuclease.

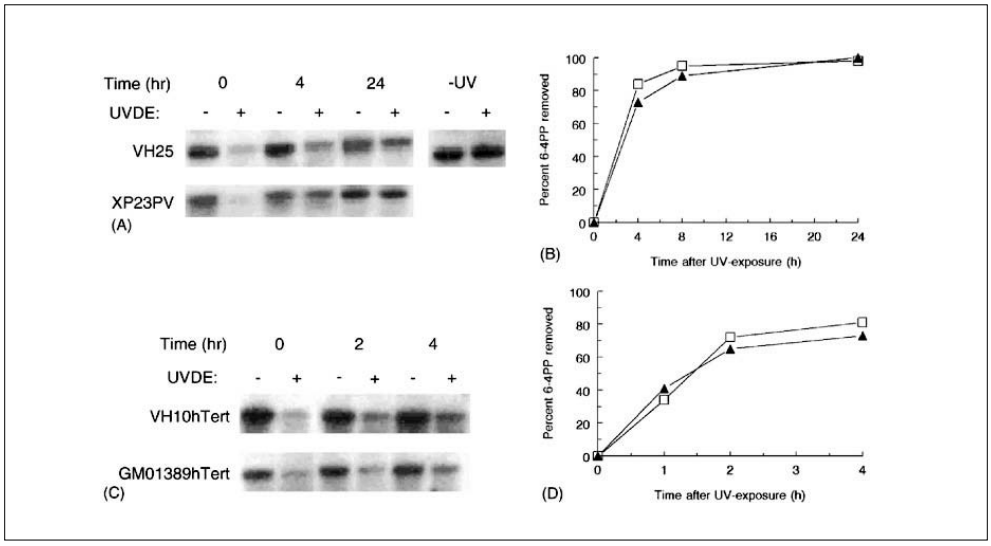

Figure 2. Normal human and XP-E cells repair 6-4PP with similar kinetics after global exposure to 30 J/m^2 UV-radiation, as determined by gene-specific analysis
Removal of 6-4PP at various times after irradiation was measured in EcoRI restriction fragments of the active ADA gene using a probe recognising both strands. After in vitro photoreactivation of CPD, the DNA was cut using the UVDE endonuclease. (A) Autoradiogram and (B) line graph showing removal of 6-4PP over a period of 24 h in (▲) primary normal human (VH25) fibroblasts and (□) primary XP-E (XP23PV) fibroblasts. (C) Autoradiogram and (D) line graph showing removal of 6-4PP over a period of 4 h in (▲) normal human hTert-immortalised fibroblasts (VH10hTert) and (□) XP-E hTert-immortalised cells.

XP-E cells. Also, 6-4PP repair at 4 h observed in primary human fibroblasts (VH25) and in hTert immortalised human fibroblasts (VH10hTert) were not different, which is in line with the observation made by Ouelette et al. (27) that hTert immortilzed cells (normal as well as XP-E) show the same UV survival as primary cells. Consistent with the findings in these biochemical experiments, immunofluorescence measurements demonstrate similar repair kinetics for 6-4PP in human XP-E fibroblasts and normal human cells after an UV dose of 30 J/m^2 (Fig. 3B and D). We note here that the results concerning repair of 6-4PP after global irradiation obtained by the two methodologies are qualitatively similar, i.e. repair in normal and XP-E cells is the same, but that quantitatively the absolute level of repair after 24 h is rather different as measured by the two methods.

3.2 Distribution of NER proteins in normal human and XP-E cells after local UV irradiation of the nucleus

The absence of clear differences in 6-4PP repair kinetics between normal human and XP-E cells suggests equally efficient incision complex formation in normal and XP-E cells. To test this hypothesis, we applied local UV irradiation to confluent human fibroblasts as described previously (9,29) taking into account that incision complex formation during the first 2 h after UV is predominantly due to repair of 6-4PP (9,31,32) (see also, Fig. 4). The distribution pattern of XPC and RPA in normal human and XP-E cells was analysed before and at various times after local irradiation (30 J/m^2). Without UV exposure, both proteins show a homogeneous distribution and strictly nuclear localization (not shown) as observed previously for NER proteins (9). In normal human cells, both XPC and RPA strongly accumulate in the UV-irradiated areas of the nucleus as fast as 5 min after UV exposure. This accumulation persists at a constant level of intensity up to 1 h after UV before slowly returning to the pre-UV pattern (Fig. 5). Since the changes in spot intensities of RPA with time are essentially similar to those of XPC in all performed experiments, only results of XPC protein are shown. In strong contrast to normal human cells, XP-E cells only display a moderate accumulation of NER proteins at damage spots 5 min after UV, while at later stages (30 min and more) after UV, hardly any accumulation is observed (Fig. 5). This indicates that in the absence of UV-DDB, formation of NER incision complexes is strongly reduced. Other

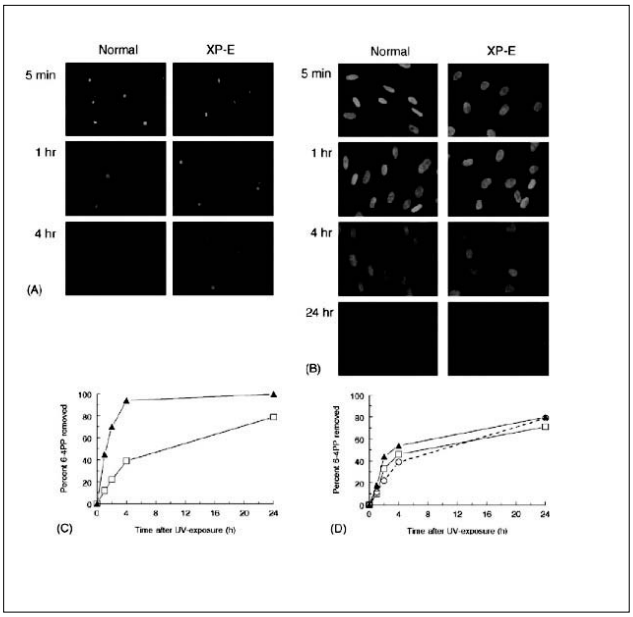

Figure 3. Accelerated 6-4PP repair in normal human fibroblasts (VH10hTert) compared to XP-E fibroblasts (GM01389hTert) after local UV irradiation, but not after global UV irradiation as measured by immunofluorescence
(A) Fluorescent immunostaining of 6-4PP (green) at various times after local UV-irradiation (30 J/m^2) through an 8 μm pore size filter. Images were taken with equal exposure times and merged with DAPI nuclear DNA staining (blue).
(B) Fluorescent immunostaining of 6-4PP (green) at various times after global UV irradiation (30 J/m^2). Images were taken with equal exposure times. (C and D) Graphs presenting the percentage of 6-4PP removed determined from multiple (>20) fluorescent images for each timepoint. (C) Removal of 6-4PP in (▲) normal human and (□) XP-E cells following exposure to 30 J/m^2 of local UV irradiation through an 8 μm pore size filter. (D) Removal of 6-4PP in (▲) normal human and (□) XP-E cells following exposure to 30 J/m^2 of global UV. The dotted line is taken from C showing removal of 6-4PP in XP-E cells which were locally UV-irradiated with 30 J/m^2 and is depicted as a reference. Signal intensities were determined by measuring the total fluorescence intensity of a spot or a nucleus, and dividing by the surface area of the measured spot or nucleus, respectively. See the Appendix for a colour version of this figure.

NER proteins XPA, XPB, XPG and ERCC1 also accumulate only marginally in spots of UV damage after local irradiation of XP-E cells (data not shown), confirming that assembly of the total NER complex is strongly reduced in quantity in the absence of UV-DDB.

Hence, our observations indicate that the complete NER machinery is able to bind to DNA photolesions in the absence of UV-DDB, but that UV-DDB positively affects the kinetics of NER complex formation.

3.3 Effect of UV-DDB on repair of 6-4PP after local UV irradiation of cells

The poor incision complex formation in the absence of UV-DDB conflicts the rather efficient repair of 6-4PP in globally irradiated XP-E cells described in this study and observed by other investigators (4,21). We reasoned that if the level of NER complex accumulation in local UV spots corresponds with the efficiency of 6-4PP repair, the strongly decreased complex formation in XP-E cells implies reduced repair of 6-4PP. Hence, we compared the repair rates of 6-4PP in locally irradiated normal human and XP-E cells at the single cell level using specific antibodies against 6-4PP. To allow direct comparison between globally and locally UV-irradiated cells and to limit experimental variations due to processing for immunofluorescence, about half of the cells on a glass slide were globally exposed whereas the remaining cells were locally irradiated and subsequently processed simultaneously, as described in Section 2.

Consistent with the profound difference in incision complex formation we find that repair of 6-4PP in local spots of UV damage differs greatly between the two cell types. From the data (Fig. 3A and C), it can be deduced that the fluorescent signal for 6-4PP in normal human cells drops rapidly in time, virtually disappearing within 4 h. In striking contrast, 4 h after UV the amount of 6-4PP in XP-E cells is only ~50% reduced compared to the initial levels. We note here that the initial levels of 6-4PP in normal and XP-E cells indicated by the immunofluorescence signal immediately after UV exposure, were virtually the same. The kinetics of accumulation of NER proteins thus coincide with the rate of repair of 6-4PP in local UV spots, strongly supporting the notion that NER complex formation shortly after UV primarily represents recruitment of NER proteins to 6-4PP. This is further supported by the finding that during the first 2 h after UV

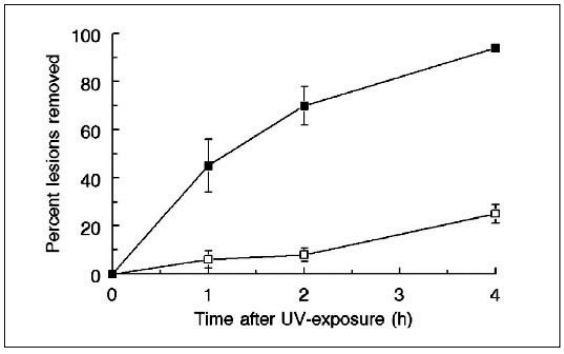

Figure 4. 6-4PP and CPD repair in normal human fibroblasts (VH10hTert) after local UV irradiation through an 8 µm pore size filter with 30 J/m² as measured by immunofluorescence
Cells were immunofluorescently labelled for 6-4PP or CPD at various times following irradiation as described in Section 2. Graphs representing percent repair of 6-4PP (■) or CPD (□) were derived from multiple fluorescent images.

irradiation repair of CPD is strongly lagging behind that of 6-4PP and actually does not amount to more than 8% removal for CPD, compared to 70% for 6-4PP (Fig. 4).

From these findings, we speculated that UV-DDB can mediate the rapid processing of only a limited number of 6-4 photolesions. To test this hypothesis, we irradiated normal human and

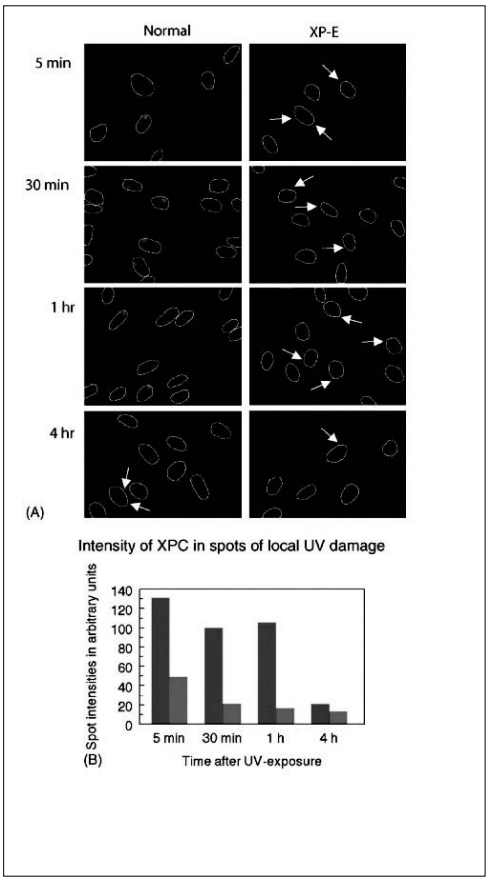

Figure 5. XPC accumulates differentially in normal human fibroblasts (VH10hTert) and XP-E fibroblasts (GM01389hTert) after local UV irradiation
Cells were UV-irradiated with 20 J/m^2 through an 8 µm pore size filter. (A) Fluorescent immunostaining of XPC (red) at various times after irradiation. Arrows indicate protein accumulations in UV-damaged spots that are poorly visible. Dotted lines outline the nuclei as determined from DAPI nuclear counterstaining (images not shown). All fluorescent images were taken with equal exposure times. (B) Bar graph showing average intensities of XPC spots at various time points after irradiation (blue bars: normal cells; red bars: XP-E cells). Spot intensities were determined by measuring the total fluorescence intensity of a spot, divided by its surface area, and corrected for background levels of fluorescence in the nucleus. For each time point, at least 20 cells were measured. The fluorescent signal measured 30 min after UV in normal human cells was set to 100%. See the Appendix for a colour version of this figure.

XP-E cells both locally and globally with a low dose of UV, i.e. 5 J/m^2 and assayed repair of 6-4PP at various times following UV using immunofluorescent labelling of 6-4PP. As shown in Fig. 6, we find that following this low-dose repair kinetics of 6-4PP are more rapid in normal human than in XP-E cells both after local and global UV irradiation. Furthermore, 6-4PP repair in normal human cells is more rapid after local irradiation than after global irradiation, whereas in XP-E cells there is no significant difference between 6-4PP repair kinetics following local or global UV. These results corroborate the idea that UV-DDB does stimulate repair of 6-4PP, but that there is a limit to the number of 6-4PP that can be rapidly repaired through UV-DDB. Interestingly, at this low UV dose, repair of 6-4PP is more rapid in normal human cells after global irradiation than in XP-E cells exposed to local irradiation, despite the fact that in the former situation more lesions are introduced (Fig. 6).

3.4 Distribution of p48 in normal human cells after local UV irradiation of the nucleus

To show that UV-DDB is part of the NER complex that is formed after UV irradiation and, thus, that accumulation of UV-DDB at locally induced UV damage spots follows the same kinetics as other NER proteins, we constructed a human cell line (MRC5) stably expressing p48-YFP. A

similar approach has been successfully applied by Fitch et al. (25,31) to investigate the recruitment of p48 to local damage. The resulting cell line termed MRC5-p48-YFP, expresses p48YFP in >75% of the cells at a moderate to high level (data not shown). Since p127 is present in excess (33), we expect that (following UV irradiation) most if not all p48-YFP to be part of UV-DDB in these cells. The distribution pattern of p48-YFP in unirradiated cells is predominantly nuclear and homogenous, as observed for other NER proteins. After local UV, p48-YFP transiently accumulates at damage spots as observed for other NER proteins such as XPC: intense spots of p48-YFP visible at 30 min after UV, become much weaker in signal 2 h post-UV, and no spots can

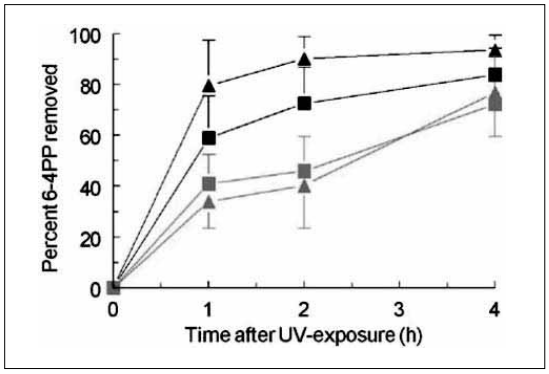

Figure 6. 6-4PP repair in normal human fibroblasts (VH10hTert) and XP-E fibroblasts (GM01389hTert) after local and global UV irradiation with 5 J/m² as measured by immunofluorescence
Cells were immunofluorescently labelled for 6-4PP at various times following irradiation as described in Section 2. Graphs representing percent 6-4PP removed were derived from multiple fluorescent images. Removal of 6-4PP in (black, ■) normal human and (grey, ■) XP-E cells following exposure to global UV. Removal of 6-4PP in (black, ▲) normal human and (grey, ▲) XP-E cells following exposure to UV through an 8 µm pore size filter.

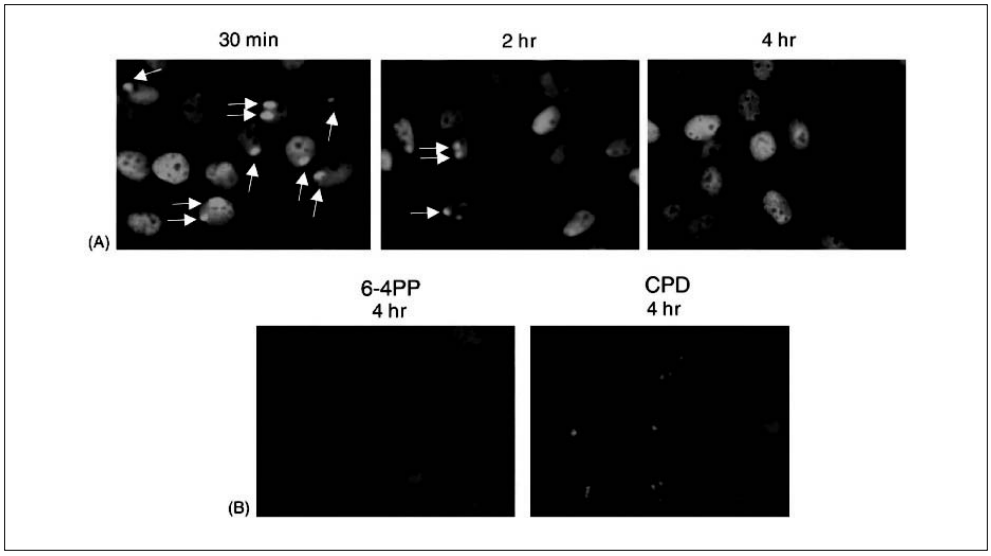

Figure 7. Formation of p48 spots after induction of local UV damage coincides with 6-4PP repair
Normal human cells (MRC5) stably expressing p48-YFP were exposed to 30 J/m² of UV through a 5 µm pore size filter.
(A) Fluorescent images of p48-YFP (green) merged with DAPI nuclear DNA staining (blue) taken at various times following irradiation. Arrows indicate sites of local UV damages. Pictures were taken with equal exposure times. (B) Fluorescent immunostaining using anti-CPD antibody (red) or anti-6-4PP antibody (red) 4 h after exposure, merged with DAPI nuclear DNA staining (blue). See the Appendix for a colour version of this figure.

be detected 4 h after UV (Fig. 7A). At 4 h post-UV, also 6-4PP have become undetectable whereas CPD are clearly detectable in locally irradiated spots (Fig. 7B), indicating that the observed accumulation of p48-YFP in local UV spots mainly takes place at 6-4PP.

To address this point in more detail, we utilized XP-A cells expressing either a CPD or a 6-4PP specific photolyase (referred to as XP-A(CPDpl) and XP-A(6-4pl) cells, respectively). As shown in Fig. 8A, CPD or 6-4PP can be specifically removed by the respective photoreactivating enzyme (25,31) and hence, this system provides an adequate tool to study the relative contribution of either type of photoproduct to the observed accumulation of NER proteins at damage spots. Fig. 8B shows typical results obtained for XPC after photoreactivation of either 6-4PP or CPD in XP-A cells. To estimate the relative affinity of XPC for 6-4PP and CPD in vivo, we utilized images that were captured with equal exposure times. A clearly reduced intensity of XPC spots can be seen when 6-4PP are selectively removed by photoreactivation compared to CPD removal in spite of the fact that CPD are present in three-fold excess over 6-4PP. Similarly, fluorescent staining of p89, the XPB component of TFIIH, also showed a clear signal upon photoreactivation of CPD whereas the signal was virtually absent upon photoreactivation of 6-4PP (data not shown). These observations confirm that 6-4PP are a better substrate than CPD for XPC and UV-DDB to bind to, as previously suggested by Fitch et al. (25,31). In order to visualize the accumulation of p48 and p127 at damage spots, XP-A(CPDpl) and XP-A(6-4pl) cells transiently transfected with both p48-YFP and CFP-p127 were locally UV-irradiated with 30 J/m^2. After removing CPD or 6-4PP by photoreactivation, cells were examined for the distribution of p48-YFP and CFP-p127 as well as immunofluorescently labelled for XPC (Fig. 8C). Under these conditions, accumulations of p48-YFP and CFP-p127 as well as XPC, are observed 1 h after UV when either all CPD or 6-4PP have been removed, suggesting that UV-DDB and XPC accumulate on 6-4PP as well as on CPD under conditions where DDB is overproduced and repair is absent (XPA).

4 Discussion

4.1 NER complex formation at early hours after UV occurs predominantly on 6-4PP

In normal human cells, the signal intensity of p48-YFP in local UV damage spots closely follows the kinetics of 6-4PP repair, in spite of the fact that UV-DDB is essential for repair of CPD in rodent and human cells. This observation suggests that 6-4PP are a stronger recognition signal for UV-DDB than CPD. Indeed, we find shortly (1 h) after irradiation that p48-YFP and CFP-p127, as well as XPC show a pronounced accumulation at 6-4PP in XP-A cells equipped with a photoreactivating enzyme for CPD. In addition, p48-YFP as well as XPC accumulate at UV damage spots in XP-A cells in which 6-4PP were removed by a 6-4PP photolyase. However, in control cells only containing low levels of endogenous p48, the signal of XPC at CPD was much weaker, while the signal of XPB was virtually absent. Fitch et al. (31), employing the same cell system, also observed a higher affinity of UV-DDB for 6-4PP than for CPD in living cells. Furthermore, they observed accumulation of XPC on damage spots in only a minority of cells analysed upon removal of 6-4PP by a photolyase, while ectopically expressed p48 was efficiently recruited to all CPD containing spots. This led them to conclude that p48 is a more efficient recognition factor for CPD than XPC. Although we agree with this statement, we conclude, based on immunostaining of the endogenous XPC protein as well as on the repair kinetics in normal human cells, that 6-4PP are a better substrate than CPD for both XPC and UV-DDB to bind to. Accumulation of both p48 and XPC at CPD in XP-A cells can be observed due to the buildup of incomplete repair complexes. A similar phenomenon is observed in normal human

cells in the presence of inhibitors of repair synthesis (unpublished results). Together, these data provide evidence that UV-DDB stimulates excision repair by direct binding to the lesion as indicated by experiments with a non-chromatin template in vitro (32) and that the accumulation of UV-DDB and other NER proteins observed shortly after local UV predominantly takes place at 6-4PP indicating a functional role of UV-DDB in 6-4PP repair, although UV-DDB also plays a crucial role in repair of CPD.

4.2 UV-DDB mediates accelerated repair of a limited number of 6-4PP

Cells proficient for UV-DDB show a more pronounced accumulation of NER proteins in spots of local UV damage, compared to cells lacking UV-DDB activity, and this enhanced complex formation coincides with an increased repair rate of 6-4PP in local UV spots. Also, after global irradiation with a low dose of UV (5 J/m^2), 6-4PP repair is more rapid in normal human than in XP-E cells. In contrast, after global exposure to a high UV dose (30 J/m^2), we find no significant difference between normal human cells and XP-E cells in the repair kinetics of 6-4PP, i.e. in this situation the accelerating effect of UV-DDB on repair is lost.

We propose the following mechanism to account for these observations. Since we applied equal UV doses in local and global irradiation experiments, the photolesion frequency per unit of DNA is the same in the irradiated areas, and hence the major difference between the

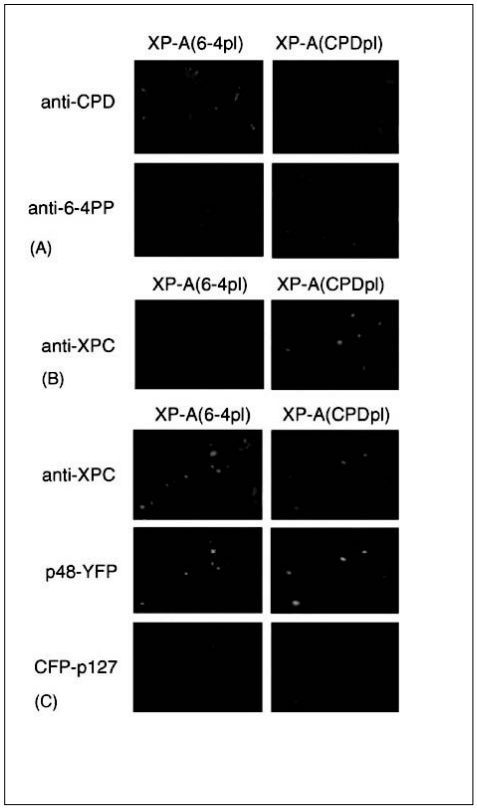

Figure 8. Accumulation of XPC, p48 and p127 in local UV-damaged spots after removal of one of the two types of UV-photolesion by photoreactivation
XP-A cells stably expressing 6-4PP photolyase [XP-A(6-4)phl)] or CPD photolyase [XP-A(CPDphl)], respectively, were irradiated with 30 J/m2 of UV through a 5 µm pore size filter, and exposed to photoreactivating light for 1 h. (A) Fluorescent immunostaining of CPD (red) and 6-4PP (red), merged with DAPI nuclear DNA staining. (B) Fluorescent immunostaining of XPC after photoreactivation of 6-4PP (left) or CPD (right) in XP-A photolyase expressing cells. Pictures taken with equal exposure times and merged with DAPI nuclear counterstaining (blue). (C) XP-A(6-4)phl or XP-A(CPDphl) cells transfected with p48-YFP and CFP-p127 expression constructs 24 h prior to local UV-exposure and 1 h photoreactivation. Fluorescent images of XPC (using anti-XPC antibody, red), p48-YFP (green) and CFP-p127 (blue), taken with exposure times optimised for image clarity. See the Appendix for a colour version of this figure.

two approaches is the total number of lesions introduced per cell. The results imply that the amount of cellular UV-DDB is sufficient to allow enhanced 6-4PP repair after the induction of a (relatively) low number of lesions, i.e. after irradiating locally with 30 J/m^2 or globally with 5 J/m^2, but insufficient to allow rapid repair of the majority of 6-4PP introduced after global irradiation with a high-UV dose. The following calculation corroborates this notion. A dose of 30 J/m^2 of UV-C light introduces approximately 2 × 10^6 CPD (30) in the (diploid) human genome (6 × 10^9 nucleotides). 6-4PP are introduced at one-third of the frequency of CPD, i.e. 7 × 10^5 in total. The typical volume irradiated after local UV through an 8 μm filter encompasses about one sixth of the whole nucleus of a human fibroblast (diameter approximately 20 μm), hence, this volume contains ~10^5 6-4PP. A comparison of the numbers of 6-4PP and the number of UV-DDB molecules, estimated to be ~10^5 (10), shows that after global irradiation with 30 J/m^2 of UV the number of 6-4PP is about seven-fold larger than the number of UV-DDB molecules; whereas, after 30 J/m^2 of local UV, this number is roughly equal. A similar calculation for global UV irradiation with only 5 J/m^2 shows that in that situation too, the number of 6-4PP is approximately the same (~8 × 10^4) as the number of UV-DDB molecules. The most plausible picture of the function of UV-DDB we envisage is that UV-DDB is present in the cell in rate-limiting amounts and is largely consumed during repair of 6-4PP. Moreover, the data suggest that UV-DDB can only function once in the NER reaction and that after recognition of a DNA lesion it becomes inactivated. Such a mechanism is supported by a recent report of Rapic-Otrin et al., who find virtually complete degradation of p48 in primary human fibroblasts in the first 3 h following 15 J/m^2 of UV (24). Other studies (20) reported similar results. As a consequence of this breakdown, most 6-4PP induced after high doses of UV are not repaired with enhanced kinetics through UV-DDB. Yet, the complete removal of 6-4PP from the genome of XP-E cells after global irradiation indicates that these lesions can also be repaired, albeit at reduced rate, through the GGR pathway in which recognition of 6-4PP solely depends on the XPC-hHR23B complex. In contrast, functional UV-DDB is essential for GGR of CPD.

Summarizing, we hypothesize that in vivo (i) 6-4PP can be repaired through a basal mechanism depending only on recognition by XPC-hHR23B; (ii) binding of UV-DDB to 6-4PP will however accelerate their repair; (iii) after binding to a 6-4PP, UV-DDB is degraded and thus cannot assist in subsequent repair of remaining 6-4PP (and CPD). This implies that if 6-4PP are introduced in molar excess to UV-DDB, most 6-4PP will be repaired through direct recognition by XPC-hHR23B, which is a relatively slow process compared to the UV-DDB-stimulated repair mechanism.

4.3 Mechanisms of action of UV-DDB

Several mechanisms might account for a stimulatory effect of UV-DDB on repair of 6-4PP. Binding of UV-DDB to a 6-4PP may result in a structure that facilitates recognition by, and stabilizes binding of subsequent repair components. NER is driven by random diffusion of single components rather than by a pre-assembled repairosome (34–36), i.e. a NER complex will be formed by the consecutive recruitment of proteins. Generally, XPC-hHR23B is assumed to be the crucial factor for initiation of the GGR process (8,9). However, in the absence of UV-DDB the interaction of XPC-hHR23B with a 6-4PP might be relatively weak and frequently subject to dissociation before binding of subsequent factors such as TFIIH, XPA and RPA can stabilize the complex as it is being formed. Consequently, only a few NER incision complexes will be formed to completion.

Two observations provide evidence for this notion. Firstly, the poor accumulation of NER proteins in local UV damage spots in XP-E cells indicates a low steady-state level of NER pre-incision complexes. Secondly, repair of 6-4PP in XP-E cells is equally fast, expressed as percent removed per time unit, after local and global UV irradiation although in the latter situation substantially more lesions are induced.

These results are consistent with a model in which both the general affinity of XPC-hHR23B for DNA and its specific affinity for 6-4PP compared to undamaged DNA is relatively low in vivo. Furthermore, several findings indicate that UV-DDB, in contrast to XPC-hHR23B, is capable of forming a stable complex when bound to a 6-4PP. The general affinity of UV-DDB for DNA is much higher (100–1000-fold) than that of XPC-hHR23B, while the specific affinity for 6-4PP is comparable (3,16). As a result of this high affinity, UV-DDB is the only factor in human whole cell extracts that binds readily to UV-damaged DNA (3). Also, it has been shown that in vivo UV-DDB can bind to photolesions independent of XPC (32). Finally, in UV-DDB-proficient normal human cells we find strong accumulations of NER proteins in local UV-damaged spots and fast repair of 6-4PP. All these data are consistent with the idea that UV-DDB, by virtue of its high affinity for DNA and 6-4PP forms a stable complex when bound to a 6-4PP. This stable state provides subsequent repair proteins – starting with XPC-hHR23B – time to detect and verify the lesion, resulting in efficient 6-4PP repair. The described scenario, in which UV-DDB directly stimulates 6-4PP repair is in agreement with the reported stimulation of 6-4PP repair by UV-DDB in an in vitro repair system employing DNA damage in naked DNA as substrate (20).

Stimulation of 6-4PP repair by UV-DDB might also involve UV-DDB-mediated local chromatin remodelling at sites of photolesions, increasing their accessibility for recognition by XPC-hHR23B. In accordance with a role in chromatin remodelling, p48 has recently been reported to interact with p300/CBP (23,24), while p48 itself bears homology to chromatin-reorganizing proteins (22). Indications that remodelling of chromatin can enhance repair come from recent studies showing that chromatin interacting proteins such as SWI/SNF (37) and HMGN1 (38) enhance NER specifically in the context of chromatin. Moreover, a recent report showed the presence of p48 and p127 together with cullin 4A in a multiprotein complex (the COP 9 signalosome) that possesses ubiquitin ligase activity (39). Not only is this complex a possible candidate to assist in chromatin remodelling by virtue of its combined ubiquitin ligase/UV-damaged chromatin binding activities (38), but also, cullin 4A has been shown to target p48 for ubiquitination and degradation (40).

Finally, UV-DDB might enhance GGR of various types of other lesions for which it displays affinity, such as CPD, *cis*-diamminedichloroplatinum(II) adducts, and nitrogen mustard, among others (3,15) by the same mechanism that accelerates 6-4PP repair.

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Chapter 10

Differential stability of early and late components of the NER complex in vivo

manuscript in preparation

10 Differential stability of early and late components of the NER complex in vivo

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Abstract

The early stages of global genome nucleotide excision repair (GG-NER) have been studied in detail, leading to the currently prevailing model wherein factors are sequentially assembled into the preincision repair complex. In contrast, the stages that follow this buildup, such as preincision complex disassembly and the DNA resynthesis and ligation steps, have been relatively poorly characterised. Here we present evidence that in vivo, the DNA polymerases δ and ε as well as DNA ligase I are involved in GG-NER, in accordance with previous in vitro findings. Investigating the disassembly of the repair complex, we find that dual incision is required for the early GG-NER proteins XPC, TFIIH, XPA and XPG to leave the complex. Upon their departure the preincision proteins are substituted by the late factors RF-C, PCNA, DNA polymerase(s) δ and/or ε and DNA ligase I. In the presence of the DNA synthesis inhibitors cytosine-β-arabinofuranoside and hydroxyurea that prevent the completion of DNA resynthesis and ligation, these postincision factors remain associated with the repair site, presumably through their stable interactions with PCNA. Finally, we find that RPA is the only early binding factor to remain associated to the repair site following dual incision, most likely assisting the formation of the DNA resynthesis complex. We speculate that the stable binding of RPA might provide a rationale for observations that in the presence of DNA synthesis inhibitors only a limited number of repair-associated breaks can be introduced in the genome, as RPA molecules tethered in postincision events are unable to fulfill their essential role in preincision repair stages.

Introduction

In humans the repair of the main ultraviolet light (UV)-induced photolesions i.e. cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP), depends strictly on nucleotide excision repair (NER). These lesions are repaired throughout the genome by the global genome repair pathway of NER (GG-NER) at different rates depending on helix distorting properties of the photolesion and its genomic location (Mullenders et al., 1986; Venema et al., 1992). Genetic defects in the GG-NER system result in the rare autosomal recessive inherited disorder xeroderma pigmentosum (XP). XP consists of 7 complementation groups (A through G) and is hallmarked by defects in GG-NER and, except for groups C and E, also in transcription coupled NER. Patients suffering from XP characteristically display severe photosensitivity, skin abnormalities and a dramatically (>1000-fold) increased risk of skin cancer.

GG-NER is a multistep repair process involving ~30 polypeptides. This minimal set of proteins required to perform complete NER has been defined using in vitro reconstituted systems (Aboussekhra and Wood, 1995; Araujo et al., 2000; Bessho et al., 1997; Mu et al., 1995) and specific roles have been assigned to the various factors involved. The XPC-hHR23B heterodimer is the damage recognition factor in GG-NER and is required for the recruitment of all following NER proteins to the damaged DNA (Sugasawa et al., 1998; Volker et al., 2001). The basal transcription factor TFIIH also plays an essential role in NER as two of its components, i.e. the proteins encoded by the XPB and XPD genes, exert their DNA helicase activities to open up the DNA helix around the lesion (Drapkin et al., 1994; Wang et al., 1995). The combined action of XPC-hHR23B and TFIIH creates short stretches of single-strand DNA (ssDNA) around the lesion that may facilitate the recruitment of ssDNA binding protein RPA. Subsequently, damage verification is performed by XPA, preventing gratuitous repair by aberrant NER complexes formed on undamaged DNA (Missura et al., 2001; Sugasawa et al., 2001; Sugasawa et al., 2002). Finally the DNA strand containing the lesion is cut at the single-strand to double-strand DNA transitions by the structure-specific endonucleases XPG and ERCC1-XPF (which cut at the 3' and 5' side of the lesion respectively) (Matsunaga et al., 1995; O'Donovan et al., 1994; Sijbers et al., 1996). Presumably, after the oligonucleotide (25-30 nt in length) containing the lesion has been removed, PCNA is loaded onto the DNA by RF-C, as is the case in DNA replication (Kelman and O'Donnell, 1995). The former factor is a stimulatory factor for DNA polymerase δ (polδ) and DNA polymerase ε (polε) (Maga and Hubscher, 1995; Podust and Hubscher, 1993; Prelich et al., 1987). In an in vitro reconstituted NER system, both polymerases are capable of DNA resynthesis across the gap using the undamaged strand as a template (Aboussekhra and Wood, 1995; Araujo et al., 2000; Shivji et al., 1995) . Finally, the remaining nick can be sealed by DNA ligase I (ligase I) (Aboussekhra and Wood, 1995; Araujo et al., 2000). Until now, evidence for a role of polδ and polε in NER in mammalian cells has been gained from studies employing in vitro NER systems. However, the recent implication in NER in vivo of DNA polymerase κ (Ogi and Lehmann, submitted) emphasises the need to confirm these findings in vivo. Finally, ligase I is subject to similar concerns as the involvement of ligase I in NER is primarily based on in vitro studies and on circumstantial evidence, such as the UV hypersensitivity of ligase I-deficient human cells (Barnes et al., 1992; Prigent et al., 1994).

In vivo, the NER factors diffuse through the nucleus as separate entities and are recruited to the lesion in a sequential manner, as opposed to working in a single multifactorial complex (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003; Volker et al., 2001). In contrast to the recruitment of NER factors, the disassembly of the complex after processing of the lesion has been less well characterised. Studies utilising in vitro systems provided evidence that XPC leaves the NER complex upon the docking of XPG into the complex, i.e. before repair is finished (Riedl et al., 2003; Wakasugi and Sancar, 1998). Wakasugi and Sancar (1998) furthermore suggested a defined sequence of steps in the disassembly of the complex: following dual incision, XPA and TFIIH leave along with the damage-containing oligonucleotide, and subsequently XPG and ERCC1-XPF are displaced when PCNA arrives. Also Riedl and co-workers suggested that the arrival of XPG directly precedes the departure of XPC (Riedl et al., 2003). In contrast to Wakasugi and Sancar, they reported the departure of all preincision NER proteins following dual incision (Riedl et al., 2003).

In this study, we focussed on three questions. First, we addressed the question which DNA polymerases and DNA ligases are recruited to the DNA damage in mammalian cells in vivo using

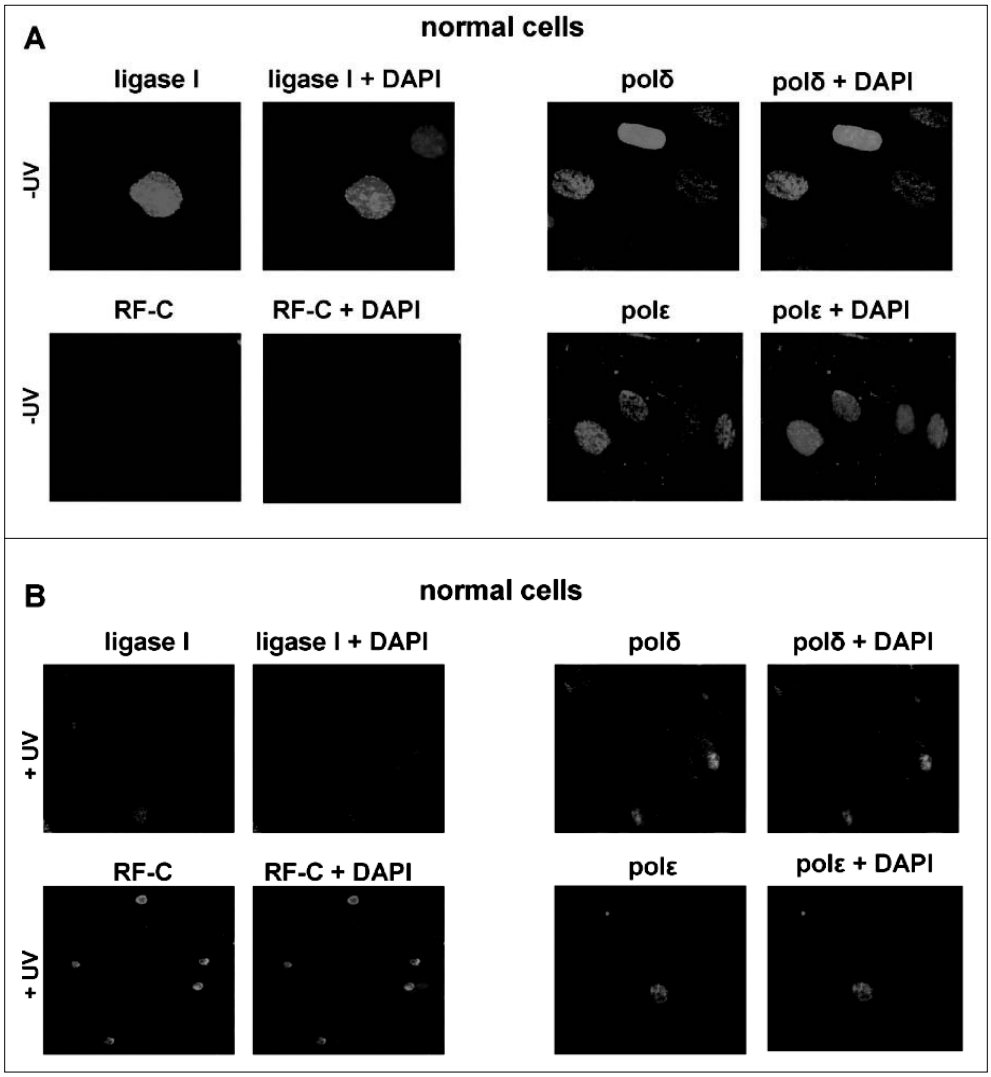

Figure 1. Involvement of RF-C, polδ, polε and ligase I in NER
RF-C, polδ, polε and ligase I were immunofluorescently labelled in normal human cells. A, RF-C, polδ, polε and ligase I show a homogeneous distribution pattern prior to UV irradiation. B, 30 min following local UV irradiation with 25 J/m^2 RF-C, polδ, polε and ligase I accumulate at sites of local UV damage. See the Appendix for a colour version of this figure.

local UV damage induction and immunofluorescent labeling of proteins (Mone et al., 2001). We additionally assessed if RF-C is recruited to sites of local UV damage, as would be expected from in vitro experiments. Second, we investigated the stability of the NER complex in vivo to elucidate whether preincision NER proteins are released in vivo from the NER complex, whether such a release depends on the capability of cells to perform dual incision, and which proteins, if any, are released from the complex. Third, we investigated the stability of the postincision NER complex, i.e. the proteins involved in DNA resynthesis, at the site of the DNA lesion.

Results

Involvement of RF-C, polδ, polε and ligase I in NER in vivo

In reconstituted NER systems, DNA repair synthesis can be executed by polδ and ε; additionally, ligase I is capable of sealing the remaining nick in the DNA and RF-C is required to load PCNA onto the DNA (Aboussekhra and Wood, 1995; Araujo et al., 2000). To investigate whether these proteins are involved in NER in vivo as predicted by the in vitro experiments, we exposed normal human fibroblasts to local UV damage and immunofluorescently labeled the proteins using specific antibodies. In unirradiated confluent (G_1) cells, the distribution pattern of polδ and polε as well as that of RF-C and ligase I was nuclear and homogeneous (Fig 1A) with some cells displaying a strong and speckled fluorescent signal. The latter most likely represent cells in S-phase as dividing cells display speckled distributions of polδ and polε related to replication foci (Fuss and Linn, 2002). In repair-proficient normal human fibroblasts, preincision NER proteins such as XPC and TFIIH are visible during a two-hour period following local UV irradiation closely mimicking the repair of 6-4PP (Volker et al., 2001; Wang et al., 2003). Following local UV irradiation, polδ and polε as well as RF-C and ligase I rapidly localised to sites of UV damage (Fig 1B) during a two-hour period following local UV irradiation.

We investigated whether the accumulation of polδ, polε and ligase I was affected in XP cells incapable of performing dual incision i.e. XP-A, XP-G/CS and XP-B/CS. Prior to irradiation the distribution pattern of the proteins in the various XP cells was homogeneous and nuclear as observed for normal human cells. In neither of these cell strains, an accumulation of polδ, polε or ligase I at UV damage was observed regardless when monitored shortly (30 min) after irradiation (Fig 2 and data not shown) or up to 8 hours following irradiation (data not shown). Thus, these findings show that only in cells that are able to progress beyond the dual incision stage of NER, polδ, polε and ligase I are recruited to the NER complex. These data also support the notion that the accumulations observed in normal cells result from bona fide NER events, as an accumulation of these proteins at stalled replication forks would lead to similar accumulations in XP cells.

Formation and dissociation of preincision NER complexes

In incision deficient XP-A cells NER proteins remain localized in spots of UV damage from 15 minutes up to 16 hours following local UV irradiation, exhibiting constant intensity of fluorescence over this time period (Fig 3) (Volker et al., 2001; Wang et al., 2003). To address the stability of NER complexes at DNA lesions in normal human cells in vivo and to analyse their composition before and after dual incision, we explore the combined use of inhibitors, local UV irradiation and immunolabeling. Combined incubation of cells with cytosine-β-arabinofuranoside (araC) and hydroxyurea (HU) efficiently reduces DNA synthesis by polδ and polε and when cells are UV-irradiated in the presence of these inhibitors, NER-associated repair patch synthesis and subsequent ligation is strongly inhibited (Mullenders et al., 1987; Smith and Okumoto, 1984).

To assess possible adverse effects of araC and HU on the early stages of NER in normal human fibroblasts, we examined the NER complex formation in locally UV-irradiated cells in the presence of inhibitors. The inhibitors did not affect accumulations of the NER proteins XPC, XPB, XPA and ERCC1-XPF at the site of UV damage when monitored 30 minutes after irradiation (Fig 4A) indicating that the entire preincision NER complex (ERCC1 is the last

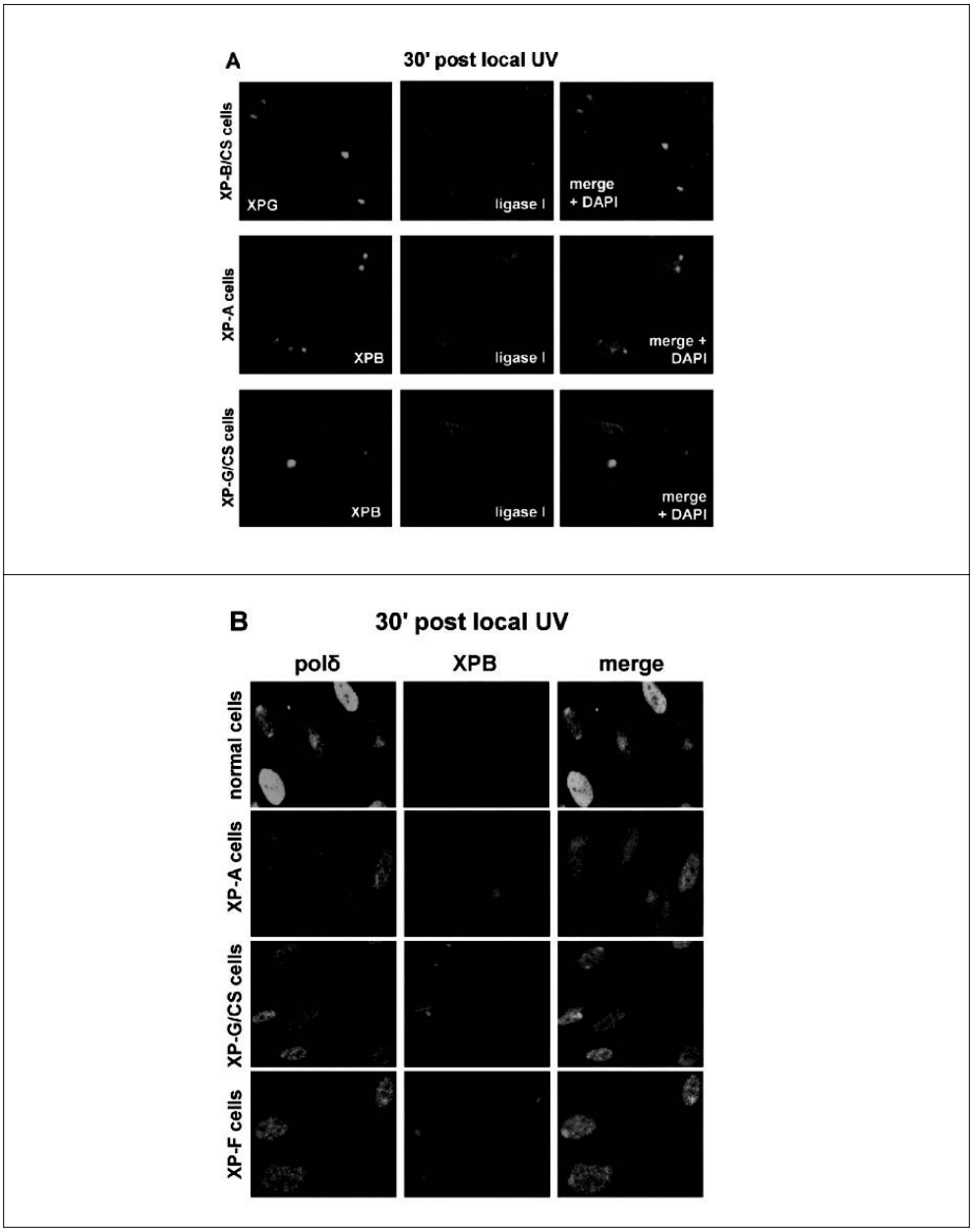

Figure 2. Differential redistribution of pre- and postincision NER proteins in normal human and XP cells
NER proteins were immunofluorescently labelled in normal human and various repair-deficient XP cells 30 min following local UV treatment with 25 J/m^2. See the Appendix for a colour version of this figure.

factor to be incorporated into the forming repair complex) can be formed in the presence of araC and HU. In addition, in araC and HU-treated cells polδ and polε also accumulated at spots of UV damage shortly following UV.

The defective ligation of the repair patch following DNA repair synthesis in the presence of araC and HU (Mullenders et al., 1987; Smith and Okumoto, 1984) could result from impaired recruitment of ligase I to the complex. However, shortly after UV irradiation we observed a clear accumulation of ligase I at UV damage (Fig 4A) in cells treated with araC and HU, indicating that this phenomenon is not caused by defective recruitment of ligase I.

Both the preincision (XPC, XPB, XPA, RPA) as well as postincision NER proteins (RPA, polδ and ligase I) remained visible for prolonged periods of time (up to 16 hours) (Fig 4B) with a fluorescence intensity similar to that displayed 30 min after UV irradiation. These prolonged accumulations of NER proteins closely resembled those observed in XPA cells in the absence of without inhibitors.

The stability of the NER complex

Preincision proteins

The data from local UV irradiation experiments showing persistent accumulations of proteins in UV damage spots in the presence of inhibitors, do not allow the conclusion that repair proteins are stably bound to DNA lesions even after incisions have been made. These results can be explained by a dynamic equilibrium of processes that assemble and disassemble NER complexes or by the recruitment of NER factors to neighbouring UV photolesions in the spot of local UV damage. We designed an experimental approach (i.e. in vivo competition experiments) to discriminate between various possibilities. In these experiments, cells were irradiated twice: starting with a global UV dose of 25 J/m^2 (a dose that saturates the global NER system) followed by a local UV dose of 25 J/m^2 or vice versa. Following the first UV irradiation cells were returned to culture conditions to allow NER complexes to accumulate. After 1 hour the second UV irradiation was administered, and cells were again incubated for 1 hour. The rationale is that proteins (especially XPC) when released from the NER complexes formed after the first irradiation would be targeted to UV damage induced by the second UV irradiation. Oppositely, if NER proteins are unable to leave the repair complexes, the distribution of a particular protein would be unaffected by the second UV irradiation. In some experiments the order of UV irradiations was reversed, i.e. an initial local UV irradiation of 25 J/m^2 was followed (after a 1-hour incubation period) by either a global UV irradiation of 25 J/m^2 or a mock-irradiation.

In normal human cells and in the absence of DNA synthesis inhibitors, NER can proceed through resynthesis and ligation, after which the NER proteins are released. To validate the experimental setup, we analysed the distribution of the NER factors XPC, XPB and XPA in normal human cells in the competition assay described above. Indeed, accumulations of the NER proteins XPC, XPB and XPA were observed at the local UV damage induced by the second UV irradiation (Fig 5), proving that the experimental protocol was capable of detecting NER proteins released from NER complexes. Experiments with XP-A cells with completely abrogated NER capacity further revealed that a global UV irradiation of 25 J/m^2 is sufficient to completely reduce the free pool of NER factors XPC and XPB by their recruitment to UV damage. In globally irradiated XP-A cells no accumulation of XPC and XPB was detected at the sites of the second (local) UV irradiation (Fig 5). Moreover, these results clearly indicate that release of proteins from NER complexes depends on NER reaching the dual incision stage.

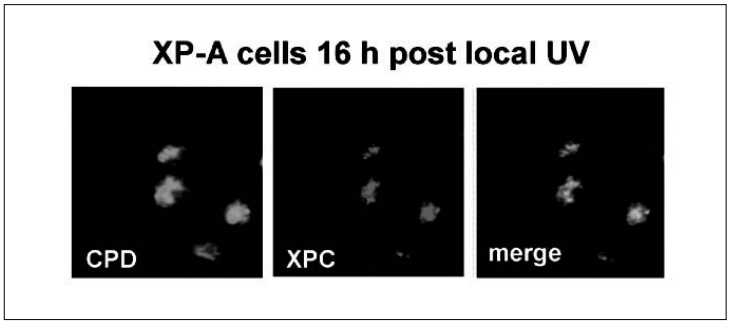

Figure 3. Sustained accumulation of XPC in NER-deficient cells
XPC and CPD were immunofluorescently labelled in XP-A cells 16 hours after 25 J/m² local UV irradiation. See the Appendix for a colour version of this figure.

Competition experiments in normal human cells in the presence of inhibitors were carried out by initial local UV irradiation of 25 J/m² followed (after a 1-hour incubation period) by either a global UV irradiation of 25 J/m² or a mock irradiation. Using this protocol, the intensity of XPC, TFIIH (p62), and XPG in local UV spots was strongly reduced compared to the spot intensity in cells that were exposed to local UV only. In fact hardly an accumulation of any of these proteins could be observed (Fig 6), indicating that preincision NER proteins are free to leave the complex despite the presence of DNA synthesis inhibitors.

RF-C, polδ, polε and ligase I
As araC inhibits elongation of the DNA ribose-phosphate chain by polδ and polε, it is conceivably that the DNA polymerases remain stalled on the DNA undergoing repair for prolonged periods of time in the presence of araC and HU. In vivo competition experiments revealed that in contrast to the preincision NER proteins (recruited to the UV damage induced by the second irradiation), polδ and polε remained confined in local UV-induced damage spots after the application of a second (global) dose of UV (Fig 6 and data not shown). Additionally, also ligase I remained present in the firstly induced UV spot (data not shown), indicating that it is not released before DNA resynthesis is completed.

RPA
RPA is the only NER protein that plays a role in both the preincision as well as the DNA resynthesis steps. Hence to understand stability of the NER complex, it is essential to know whether RPA remains immobile during the entire NER reaction, or whether it dissociates following dual incision and reassociates with the complex during the subsequent formation of the DNA synthesis complex. The competition experiments in normal human cells in the presence of araC and HU and immunofluorescently labeled RPA revealed that in striking contrast to the other preincision NER proteins, RPA remained accumulated in the firstly introduced local UV damages (Fig 6). This indicates that RPA is not released from the site of the damage like the other NER proteins but instead remains firmly bound, presumably to the short stretch of ssDNA.

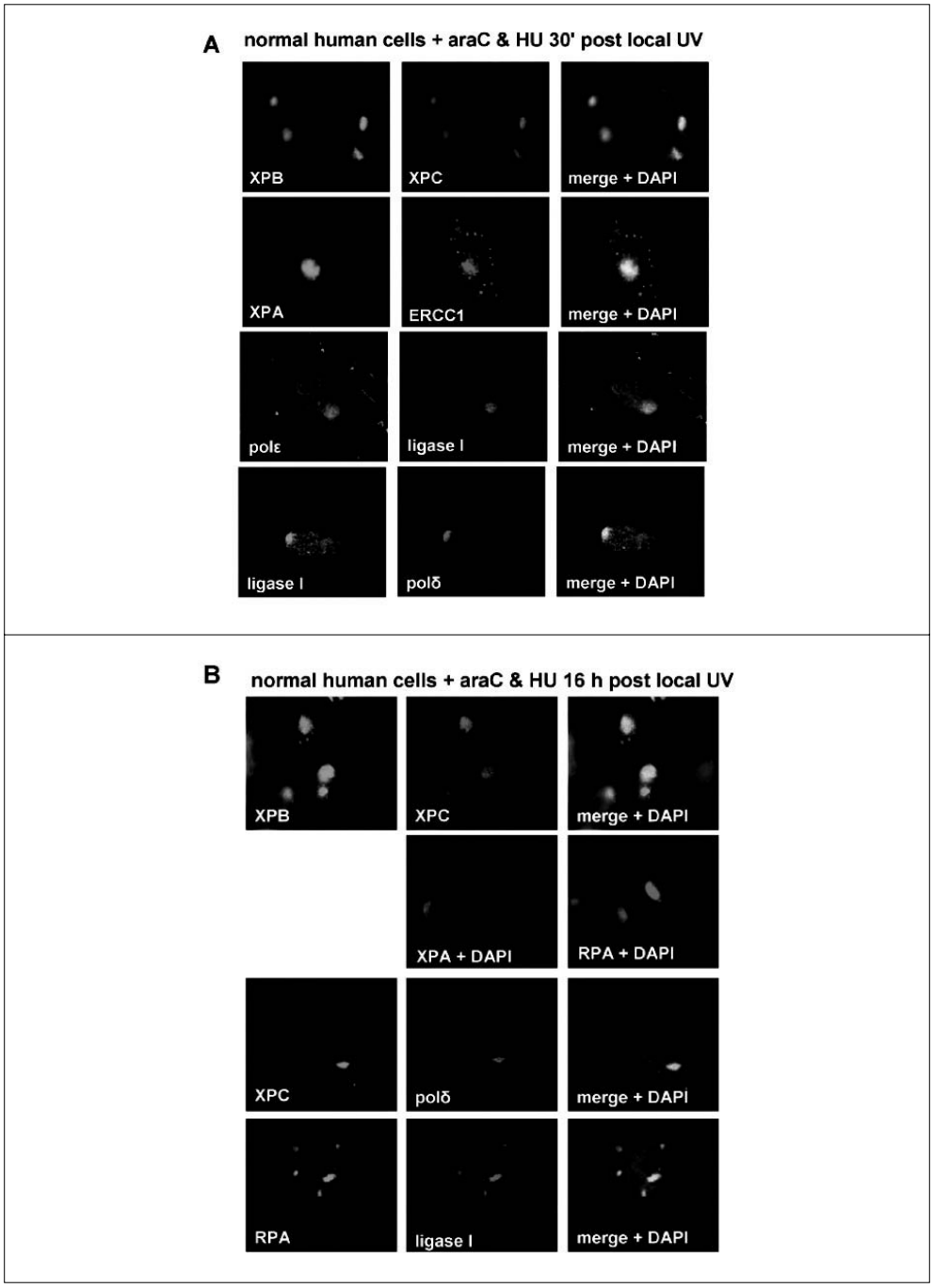

Figure 4. Rapid and prolonged accumulation of NER proteins in normal human cells in the presence of araC and HU
NER proteins were immunofluorescently labelled following local UV irradiation with 25 J/m2 in the presence of araC and HU. A, 30 min after UV. B, 16 hours after UV. See the Appendix for a colour version of this figure.

Discussion

Involvement of RF-C, polδ, polε and ligase I in NER in vivo

Previous studies utilising in vitro repair assays (Aboussekhra and Wood, 1995; Araujo et al., 2000; Shivji et al., 1995) identified polδ and polε as the principal DNA polymerases in NER. The results described in this study provide the first direct experimental evidence that polδ and polε are involved in NER in vivo. Both polymerases accumulate readily at sites of local UV, suggesting that both polymerases might carry out repair synthesis after UV irradiation and that neither of the polymerases is recruited preferentially. The resolution of fluorescent microscopy does not allow distinguishing whether both polymerases are simultaneously present at a single repair site, or as single entities at closely spaced sites. Although recruitment per se does not prove that both DNA polymerases are indeed engaged in DNA repair synthesis, the results are consistent with reduced UV-induced repair synthesis by aphidicolin, an inhibitor of DNA polymerases δ and ε (Smith and Okumoto, 1984). Also, studies with yeast strains carrying mutations in polδ and polε support a role of both polymerases in NER (Budd and Campbell, 1995). Yet we cannot rule out that NER utilises several PCNA-dependent DNA polymerases in NER including putative translesion synthesis polymerases (Ogi and Lehmann, submitted) Moreover, our results establish the involvement of DNA ligase I in NER in vivo, further corroborating the existing in vitro data (Aboussekhra and Wood, 1995; Araujo et al., 2000; Shivji et al., 1995). Finally, this study confirms the involvement of RF-C in NER in vivo where it is likely involved in the loading of PCNA onto the DNA at the site of the excised oligonucleotide.

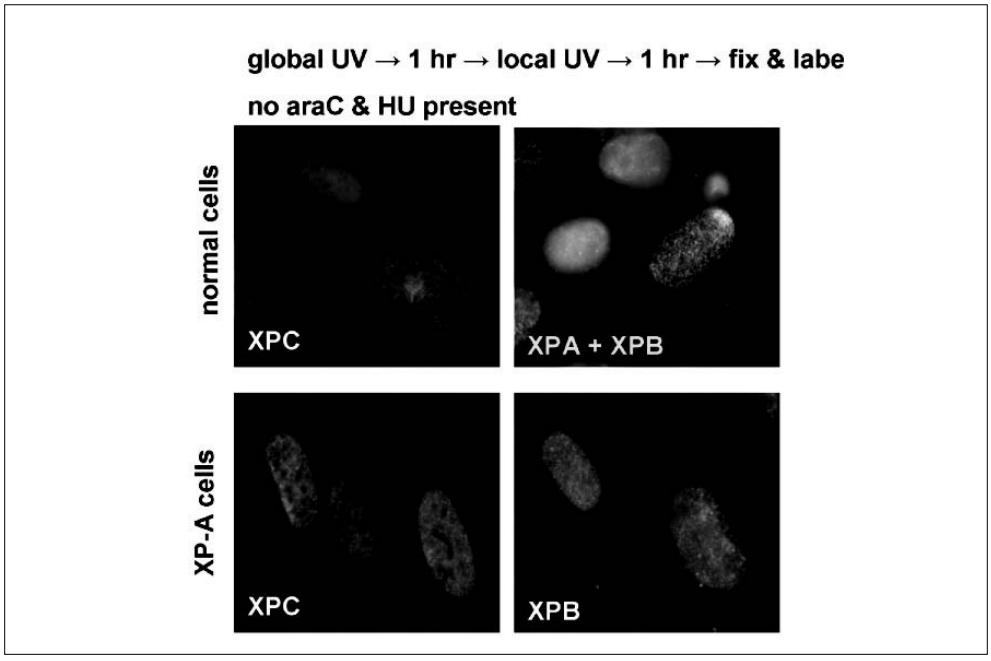

Figure 5. Stability of preincision NER proteins in the NER complex in the absence of araC and HU
Normal human or XP cells were globally irradiated with 25 J/m², incubated for 1 hour, locally irradiated with 25 J/m² and incubated for another 1 hour before NER proteins were immunofluorescently labelled. See the Appendix for a colour version of this figure.

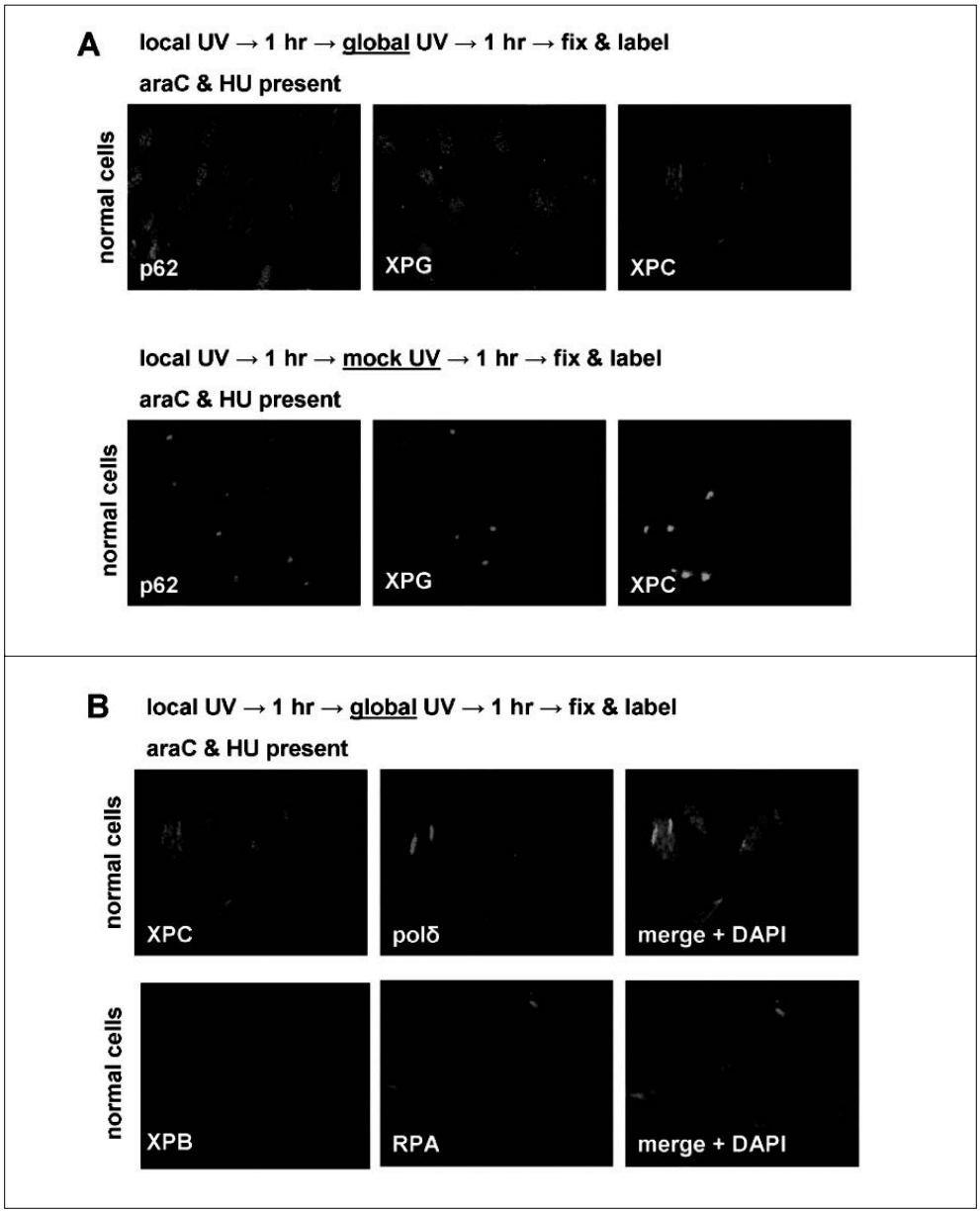

Figure 6. Stability of preincision and postincision NER proteins at the site of DNA lesions in the presence of araC and HU
Normal human cells were locally irradiated with 25 J/m², incubated for 1 hour, globally irradiated with 25 J/m² and incubated for another 1 hour before NER proteins were immunofluorescently labelled. A, preincision NER proteins are tethered away from sites of primary UV damage by competition with the secondary UV lesions. B, NER proteins involved in the postincision stages remain stably associated with the primary UV lesions. See the Appendix for a colour version of this figure.

In XP mutant cells lacking incision activity, no accumulations of polδ, polε or ligase I were observed. These findings fit well with previous data demonstrating that PCNA does not associate with chromatin nor accumulates at sites of local UV damage in NER-deficient cells (Aboussekhra and Wood, 1995; Green and Almouzni, 2003; Katsumi et al., 2001; Miura and Sasaki, 1996). PCNA is required for polδ to execute its function and also interacts with ligase I (Tom et al, 2001). Taking all data together, we propose that all post-incision proteins involved in NER are recruited in a coordinated fashion following (and depending on) dual incision, with PCNA as the key mediator. Meanwhile it is also clear that RPA, which accumulates at UV damage sites in cells from various XP groups (Rademakers et al., 2003) is not capable to recruit these proteins.

Stability of the NER complex

Results of in vitro studies (Riedl et al., 2003; Wakasugi and Sancar, 1998) suggest that completion of NER might require preincision NER proteins (such as XPC) to leave the NER complex. To address this question in vivo, we treated normal human cells with the DNA synthesis inhibitors araC and HU, that efficiently block repair synthesis after UV irradiation (Mullenders et al., 1987; Smith and Okumoto, 1984). Following a single local UV irradiation of normal human cells treated with araC and HU, spots of NER proteins persisted for up to 16 hours (fig 4B). In XP-A cells a similar result was obtained but without requiring the presence of inhibitors. These findings indicate that the entire NER complex remains stable at the site of a lesion until the resynthesis step is completed or alternatively, that any released protein could be tethered to other sites of UV damage inside the same local UV spot. However, subsequent in vivo competition experiments uncovered clear differences between the stability of the NER complexes in XP-A cells and in normal human (in the presence of araC and HU). The NER complex in XP-A cells was stable, whereas the NER complex in normal human cells in the presence of inhibitors appeared to be unstable. Hence, in the absence of the XPA protein, the core NER proteins XPC-HR23B, TFIIH, XPG and RPA assemble into a stable complex. These observations together suggest that dual incision is the key determinant for the release of preincision NER proteins. The findings that in XP cells the later stage NER proteins (PCNA, polδ, polε and ligase I) are not recruited to sites of damage furthermore suggest that dual incision is also strictly required for this recruitment.

XPC and XPG both remain present in UV spots in XP-A cells, suggesting that both proteins are present in the same complex simultaneously or reside in stable but different populations of NER complexes containing either XPC or XPG. The latter is unlikely because this situation would almost certainly require one or both these proteins to be mobile and to leave the complex at some time. The stable complex formation in XPA cells conflicts with results from in vitro studies indicating that XPC leaves the NER complex upon the arrival of XPG (Riedl et al., 2003; Wakasugi and Sancar, 1998). Measuring the in vivo dynamics of XPC-GFP after UV, it was found that XPC has a shorter retention time in the NER complex than most other proteins (approximately 2.5 min v. 4 min)(Politi et al., 2005). Based on our data we propose the following sequence of events. After recruitment of the endonucleases XPG and ERCC1-XPF to the complex, dual incision takes place. This results in the release of XPC, possibly together with the damaged oligonucleotide. Repair factors such as TFIIH, XPA and XPG remain bound at this stage, explaining the difference in measured retention time between XPC and the other factors. In support of this model is the observation that XPG has been reported to interact with PCNA (Gary et al., 1997; Warbrick, 1998) suggesting an assisting role for XPG in the recruitment of PCNA.

As mentioned above, the preincision NER complex in normal human cells in the presence of inhibitors appeared to be unstable. In contrast, proteins that operate in DNA resynthesis, i.e.

polδ, polε and ligase 1, remain present at the site of the initial UV damage when normal human cells are challenged by a second UV irradiation in the presence of inhibitors. The most likely explanation for these observations is that these proteins are bound to PCNA. Since PCNA will probably dissociate only after the completion of repair synthesis and ligation (impaired in the presence of the inhibitors), the entire DNA resynthesis complex will remain assembled at the site of repair.

RPA: a role in limiting the total number of incisions by NER?

Our findings clearly indicate that RPA is the only NER protein to remain associated at the site of repair following dual incision, consistent with the findings of in vitro studies by Riedl et al. (2003). The persistence of RPA in local UV spots in the competition experiments implies that RPA does not leave following dual incision to reassociate when DNA synthesis starts, but instead remains bound to the DNA throughout the reaction. This observation could provide an explanation for several phenomena. As described above, locally UV-irradiated normal human cells treated with inhibitors, display accumulation of proteins at spots of UV damage for prolonged periods of time (over 16 hours). Yet, NER complexes formed under these conditions are unstable, i.e. factors such as XPC and TFIIH can initiate new NER events after being released. If these initiations would all result in complete repair, accumulated NER proteins are expected to disappear over time. The observed persistence of accumulated NER proteins suggests that one or more NER factors become limiting. Given our observations the likeliest factor to become limiting is RPA. This protein remains bound following dual incision to engage in DNA resynthesis, a process that will not reach completion in the presence of inhibitors. Thus, over time all RPA will be sequestered in complexes stalled at the DNA repair synthesis step of NER, hence preventing it from executing its essential role in dual incision (Coverley et al., 1991). We propose that the accumulated NER proteins observed at extended time periods after UV represent components of two types of complexes: one that has completed dual incision and has released its early NER proteins except RPA, and one that has formed in the absence of RPA and therefore will never reach dual incision. Alternatively, one or more factors crucial to the preincision complex could become exhausted by degradation or inactivation, but we have no indications that this is the case.

Interestingly, the number of incisions made by NER in the presence of araC and HU reaches a maximum as early as one hour after irradiation (Berneburg et al., 2000; Mullenders et al., 1985) indicating that NER in the presence of inhibitors is non-catalytic, i.e. the NER system can only incise a limited number of times. A possible explanation for this limited incision capacity of NER is that one or more NER factors become 'trapped' inside NER complexes which are stably bound at lesion sites; our data suggest this factor is RPA.

Materials and methods

Cell culture

The primary diploid human fibroblasts were derived from a normal individual (VH25), and xeroderma pigmentosum patients (XP25RO, complementation group A; XP131MA, complementation group B/CS; XP24KY, complementation group F; XPCS1RO and XPCS1LV, complementation group G/CS). The cells were seeded on glass coverslips coated with Alcian Blue (Fluka) and grown in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics at 37 °C in a 2.5% CO_2 atmosphere.

UV irradiation
UV irradiation was performed essentially as described (Mone et al., 2001). Briefly, prior to irradiation, medium was aspirated and kept. Cells were rinsed with PBS and exposed to UV radiation with a Philips TUV lamp (predominantly 254 nm). For local UV irradiation, the cells on coverslips were covered during the irradiation with an isopore polycarbonate filter with pores of 5 or 8 μm diameter (Millipore, Badford, MA), after which the filter was removed. Following irradiation, the medium was added back to the cells and cells were returned to culture conditions. Cytosine-β-arabinofuranoside (Fluka) and hydroxyurea (Fluka) when employed were added to the medium 30 minutes prior to irradiation and remained present throughout the time course of the experiment. Final concentrations were 10 μM for cytosine-β-arabinofuranoside and 10 mM for hydroxyurea.

Fluorescent labeling
Immunofluorescent labeling was performed essentially as described (Volker et al., 2001). Briefly, cells were washed with cold PBS, fixed and lysed for 15' on ice in PBS with 2% formaldehyde and 0.2% triton X-100 and washed again with cold PBS. Cells were then incubated with 3% bovine albumin in PBS for 30' at room temperature. Antibodies were incubated for 2 hours (primary antibody) and 1 hour (secondary antibody) at room temperature in washing buffer (PBS + 0.05% tween-20). Following each antibody incubation cells were washed three times for 5 minutes with washing buffer. Cells were mounted in Aqua/polymount (Polysciences Inc., Warrington, PA) containing DAPI (1.5 μg/ml) to counterstain the nuclear DNA.

Antibodies
Primary antibodies employed in this study were affinity-purified mouse IgG monoclonal anti-RPA70, a gift from Dr. H.P. Nasheuer (National University of Ireland, Galway, Ireland); mouse monoclonal anti-XPA (Abcam, Cambridge, United Kingdom); mouse IgG monoclonal anti-XPB and p62, a gift from Dr. J-M. Egly (IGMC, Illkirch, France); affinity-purified rabbit IgG polyclonals anti-XPC and anti-ERCC1, gifts from H. Odijk and Dr. W. Vermeulen (Erasmus MC, Rotterdam, The Netherlands); mouse IgG monoclonal anti-XPG, a gift from Dr. R.D. Wood (UPCI, Pittsburg, PA); mouse monoclonal anti-RFC1, a gift from Dr. B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); mouse monoclonal anti-polε, a gift from Dr. S. Linn (UC, Berkeley, CA); mouse monoclonal anti-polδ (Santa Cruz), rabbit IgG polyclonal anti-ligase I, a gift from Dr. A. Montecucco (Università di Pavia, Pavia, Italy) and mouse monoclonal anti-thymine dimer, a gift from Dr. O. Nikaido (Kanazawa University, Kanazawa, Japan). Utilized secondary antibodies are: donkey anti-rabbit IgG and donkey anti-mouse IgG conjugated with FITC; goat anti-rabbit IgG and goat anti-mouse IgG conjugated with Cy2; and goat anti-rabbit IgG and goat anti-mouse IgG+IgM conjugated with Cy3. All secondary antibodies were obtained from Jackson Laboratories (Westgrove, PA) and used according to the manufacturer's instructions.

Microscopy
Fluorescence images were obtained with a Zeiss Axioplan 2 fluorescence microscope equipped with an AttoArc HBO 100W adjustable mercury arc lamp and fitted with appropriate filters for FITC/Cy2, Cy3 and DAPI. Digital images were captured with a cooled CCD camera (Hamamatsu, Japan) and processed with the Metasystems ISIS software package (Metasystems, Altlussheim, Germany).

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Summary

Summary

DNA, the physical carrier of our genetic information, is not as inert as one imagines or as one might want it to be. It deteriorates spontaneously, and it is also constantly threatened by both exogenous and endogenous chemical and physical agents. If the cell does not deal appropriately with damage to its DNA, the consequences may be serious. One possible effect is the stalling of DNA and RNA polymerases on the lesions. A stalled polymerase physically prevents a cell from replicating its DNA, and hence from dividing; this could lead to the cell's death. Another possibility is that the wrong nucleotide is inserted opposite the lesion, introducing mutations that can cause malfunctioning proteins and eventually could result in cancer.

Several systems have evolved that counteract the harmful effects of damaged DNA. Generally, the cell will stop its cell cycle, to prevent the replicative polymerases from attempting to copy the damaged DNA. At the same time, this gives the cell time to try and repair the damage, using its DNA repair pathways. Sometimes, the DNA may be damaged to such an extent that the cell assesses repair to be impossible; in such cases, the cell may commit to the 'ordered selfdisassembly program' known as apoptosis. By undergoing apoptosis and removing itself, the cell prevents more serious damage (e.g. the formation of cancer) to the entire organism. **Chapter 1** briefly describes these responses with a focus on the DNA repair pathways, outlining their mechanism of action and the main factors involved.

The research in this thesis is focused on the DNA repair system nucleotide excision repair (NER). The NER pathway is capable of repairing a wide variety of DNA lesions, including photolesions caused by the ultraviolet component of sunlight (cyclobutane pyrimidine dimers, CPD and pyrimidone 6-4 pyrimidone photoproducts, 6-4PP), small DNA adducts caused by the anticancer drug cisplatin, and large adducts introduced by the aromatic carbohydrates found in burnt food and cigarette smoke. At the molecular level, the mechanism of NER consists of several steps: recognition of the damaged DNA; unwinding of the DNA double helix immediately surrounding the lesion; and incision of the DNA strand containing the damage on both sides of the lesion, after which the oligonucleotide containing the damage is removed. Then, using the undamaged opposite strand of DNA as a template the gap is filled and subsequently sealed, resulting in fully restored DNA.

NER has been extensively researched; **chapter 2** provides an overview of the basic concepts of NER, including a brief sketch of one of the two subpathways of which it is composed, i.e. transcription-coupled repair (TCR). TCR couples repair of DNA lesions to RNA polymerases stalled on damaged DNA, allowing the lesions to be removed rapidly so that they obstruct transcription as short as possible. The second pathway of NER is global genome repair (GGR) which indiscriminately removes lesions from the entire genome; it is elaborated on in chapter 3. Also discussed in chapter 2 are the consequences of defective NER as evident from several inherited disorders. People with a defect in GGR suffer from the disorder called xeroderma pigmentosum (XP). XP patients display several features resulting from their repair defect, but the most striking (and most fundamental) of all is an extremely increased risk of developing skin cancers at sun-exposed parts of their skin, underlining the importance of GGR in preventing cancer. On the other hand, people that suffer from Cockayne syndrome (CS) have a defect in TCR; in contrast to XP, CS does not comprise cancer-proneness but instead CS patients display (amongst other features) growth defects and mental retardation. These features are ultimately linked to the defect in transcription that is described in detail in chapter 4. Finally chapter 2

describes two basic concepts upon which our current thinking of NER is based: the idea that the proteins involved in NER are recruited to the lesion sequentially (as opposed to all at once), and the 'bipartite' model for damage recognition that proposes that NER does not recognise a DNA lesion in a single step but in two, each step sensing a different characteristic of the damaged DNA: disturbing of the base stacking in the DNA helix and the presence of a chemical alteration in the DNA.

As mentioned, **chapter 3** then provides a detailed account of the major NER pathway, i.e. GGR. The proteins involved, their respective roles and their interactions are extensively discussed. First, the two so-called 'damage recognition factors', UV-DDB and XPC-hHR23B are covered. While XPC-hHR23B is generally considered the factor necessary to initiate GGR by detecting damaged DNA, UV-DDB may play an assisting role in this recognition step. UV-DDB and its role in repair of 6-4 photoproducts is elaborated on in chapter 9. The next factor to be addressed is the multifunctional, multisubunit TFIIH. Initially thought to be merely necessary to open up the DNA to provide access to subsequent proteins, it has become clear that in fact, TFIIH plays a very active role in regulation of these proteins and perhaps also assists in recognition of the damage. Then, XPA is covered. At one time proposed to be the damage recognition protein (assisted by RPA), it is now clear XPA is involved in the second step in the bipartite NER model: verification of the presence of a lesion in DNA. A model is presented in which XPA accomplishes this through the tight regulation of the subsequent dual incision steps; XPA might cooperate closely with TFIIH in this phase of NER. As mentioned, RPA was once thought to be involved in damage recognition together with XPA. However, it has been established that RPA, due to its extraordinary high affinity for single-strand DNA, plays an important role in NER in stabilising the undamaged strand when the helix has been opened up and the damaged piece of DNA has been removed. Additionally, RPA is of importance for the regulation of the two endonucleases XPG and ERCC1-XPF that make the nicks to remove the damaged DNA. The order of binding to the NER complex of XPA, RPA and XPG is not clear. Evidence for and against several binding orders is discussed, as well as the proposal that in fact, these proteins may not bind in a specific but in a random order. ERCC1-XPF is the last factor to be incorporated into the preincision NER complex before dual incision occurs. By the time the oligonucleotide containing the damage is removed, all preincision NER proteins are removed from the DNA apart from RPA and possibly XPG (see also chapter 10); unclarity still exists whether some proteins may leave the complex earlier. Especially XPC-hHR23B has in a number of reports been found to leave the NER complex around the same time that XPG enters it. To the single-strand gap (with RPA bound to it), proteins are then recruited that are also involved in DNA replication: the ring-shaped clamp for DNA polymerases, PCNA; RF-C, which loads PCNA onto the DNA; the DNA polymerases delta and/or epsilon, and the DNA ligase I.

Transcription interacts with NER in one more way that is not discussed in chapters 1 through 3. Upon the infliction of DNA damages that are targeted by TCR (including the UV-induced photolesions), transcription is reduced; normally this inhibition is released when the lesions are removed. Strikingly, also transcription of genes that are not damaged is reduced. As yet it is unknown what causes this effect. **Chapter 4** discusses possible mechanisms for both the origin of the inhibition and how undamaged genes may be affected. Fundamental to the inhibition is the hyperphosphorylation of RNA polymerase II, resulting in its inactivation in transcription initiation. In CS cells this hyperphosphorylation is not reversed even when damages have been removed, and the resulting permanent reduction in transcription is thought to be the root cause of many of the CS patients' characteristics.

The final introductory chapter, **chapter 5**, puts NER in the context of the nucleus, where it has to deal with DNA being packaged into chromatin, which in itself has an inhibitory effect on NER. Chromatin remodelling can however alleviate much of this inhibition, both by 'shuffling' histones around on the DNA and by modifying the histones so that the DNA is less efficiently packed by them. Proteins involved in either mechanism that have been found to enhance NER, as well as proteins that in some other way reduce the restrictive effects of chromatin on NER are discussed in this chapter. Finally, also the post-NER chromatin restoration is highlighted.

Most of the research into NER has been performed *in vitro*, i.e. using cell extracts and/or purified proteins. The research described in this thesis, on the other hand, was mostly done in intact cells. The major advantage of using intact cells is that there are no variable external circumstances (e.g. salt concentrations, activity of purified proteins) that can influence the outcome of experiments.

In **chapter 6**, the order of binding of several NER proteins to the NER complex is described. To be able to study this, we developed a method to study NER and other processes interacting with UV-induced DNA damage. In this elegant and simple method, cells grown on coverslips are covered with a polycarbonate filter with pores that leave a small area of the nucleus uncovered. Upon UV irradiation, the polycarbonate blocks all UV so that cell nuclei are only exposed to UV directly underneath the pores (this method is therefore usually referred to as 'local UV irradiation'; it is schematically depicted in chapter 8, figure 5). Processes that react to UV lesions can be visualised in these spots using for instance indirect immunofluorescence. We firstly found that XPC is the first core NER protein to bind to UV-lesions and thus, to be the damage recognition protein in GGR. XPA, the other candidate for this role, was shown to bind later, after the recruitment of TFIIH. These findings corroborated in vivo what had been reported by others in vitro. Secondly, XPA and XPG were found not to depend upon one another for their recruitment to the NER complex and finally, the recruitment of ERCC1-XPF was found to depend on XPA, but not XPG.

Chapter 7 describes the method of 'local irradiation' in greater detail. Furthermore, it was used to study the relation between UV irradiation and the abovementioned transcription inhibition. We found that the inhibition was restricted to areas of the nucleus that are UV-irradiated while in the remainder of the nucleus, transcription carried on as normal. We concluded that the signal for transcription inhibition is not propagated by a factor that can freely diffuse through the nucleus, such as TFIIH (which was a candidate factor).

A technique that is being used extensively in the research in live cells is the coupling of a protein species of interest to green fluorescent protein (GFP). This enables one to follow the proteins in the living cell using a confocal microscope. In **chapter 8**, the behaviour of GFP-tagged XPA is described. Measuring its rate of diffusion enabled us to determine that XPA was not travelling through the nucleus as part of a large protein complex such as a 'repairosome' (i.e., a large stable complex containing most repair factors), providing more evidence for the sequential assembly model described in chapter 2. We also found that after local UV irradiation RPA was incorporated into the NER complex even in the absence of XPA, overturning a number of older reports claiming that a complex of XPA and RPA was functional in binding to DNA lesions.

The UV-DDB protein appears only to be required for the repair of the UV-induced CPD, while repair of the other major type of UV-induced photolesions, the 6-4PP seemed hardly or not affected in cells lacking UV-DDB. This was especially puzzling as UV-DDB binds with more affinity to the latter type of lesion than the former. Using a variety of methods, in **chapter 9** we

showed that UV-DDB could in fact have a large impact on the repair of 6-4PP. However because of the peculiar characteristic of UV-DDB to be degraded during repair its effect was limited to a small number of lesions. If a small number of 6-4PP was introduced, their repair was greatly accelerated by the presence of UV-DDB; if however a high UV dose was used, this effect was lost. In that case, repair of 6-4PP was carried out independently of UV-DDB, because in contrast to CPD, 6-4PP can be recognised by XPC and subsequently repaired.

In vitro research has suggested that DNA polymerases delta and epsilon, as well as DNA ligase I, are involved in the final stages of NER: DNA resynthesis and ligation. In **chapter 10**, using local UV irradiation and immunofluorescence, we found strong evidence that this is also the case *in vivo*, as both DNA polymerases delta and epsilon and DNA ligase I are found to accumulate at the sites of UV damage. Furthermore, irradiating cells twice – once globally and once locally – we were able to determine whether NER proteins leave the complex, and at what stage of the reaction. We found that all preincision factors except RPA dissociate following dual incision; subsequently the resynthesis and ligation proteins were recruited. In the absence of dual incision the preincision complex was stable, and no recruitment of DNA polymerases or DNA ligase could be observed.

Samenvatting voor de leek

Samenvatting voor de leek

Onze erfelijke eigenschappen liggen opgeslagen in het DNA in elk van onze cellen. DNA is opgebouwd uit lange strengen nucleotiden; elk nucleotide is een 'letter' uit de genetische code. Er zijn vier soorten nucleotiden in DNA die we aanduiden met de letters A, T, C en G. Drie miljard van deze nucleotiden coderen alle erfelijke eigenschappen van de mens. In het genoom (alle nucleotiden bij elkaar) ligt elke 'letter' altijd tegenover dezelfde andere letter: A tegenover T en C tegenover G; dit maakt een totaal van zes miljard nucleotiden in drie miljard 'baseparen'. De twee lange strengen DNA zijn om elkaar heen gedraaid als een soort wenteltrap, en dit levert het beeld van de spreekwoordelijke 'DNA-helix' op. Het voordeel van deze manier van organiseren is dat alle informatie twee keer aanwezig is, één keer in elke streng van het genoom. Het kopiëren van DNA ('replicatie') bijvoorbeeld is daardoor in principe eenvoudig: de twee strengen worden in feite uiteengeritst en tegenover elk nucleotide plaatst de cel het corresponderende nucleotide; het eindresultaat is twee kopieën van het genoom, waarna de cel zich in tweeën kan delen. Om eiwitten te maken wordt er eerst een kopie van het DNA gemaakt in RNA in een proces dat transcriptie heet. Dit RNA wordt daarna vertaald naar eiwit (dit proces heet 'translatie'), waarbij elk blok van drie 'letters' één bouwsteen voor een eiwit codeert; een deel van het genoom dat de informatie bevat voor het maken van één eiwit wordt een gen genoemd. Een fout in het DNA kan dus leiden tot een fout in een eiwit; dit is een belangrijke onderliggende oorzaak voor het ontstaan van kanker.

Het is dus belangrijk dat er geen fouten in ons genomisch DNA sluipen maar dat is makkelijker gezegd dan gedaan. DNA is namelijk niet zo stabiel als men zich meestal voorstelt. Het DNA kan spontaan uiteenvallen en wordt bovendien beschadigd door chemische stoffen – zowel van buitenaf als door de cel zelf geproduceerd – en straling. Als de cel beschadigingen aan zijn DNA niet adequaat oplost, kan dat ernstige gevolgen hebben. Eén van de mogelijkheden is dat de polymerases, die transcriptie en replicatie uitvoeren, vastlopen op de beschadiging in het DNA, omdat ze niet geschikt zijn om beschadigd DNA te 'lezen'. Een vastgelopen polymerase is een belemmering voor de cel om zijn DNA te kopiëren voordat de cel kan delen en kan ertoe leiden dat de cel doodgaat. Ook kan een beschadigd nucleotide verkeerd gelezen worden tijdens replicatie en zo leiden tot een soort 'drukfout' in het DNA (een 'mutatie'). Zoals gezegd kan dat leiden tot het ontstaan van kanker.

Gelukkig zijn er verschillende systemen geëvolueerd die de schadelijke effecten van beschadigd DNA tegengaan. Ten eerste zal de cel vaak de zogenaamde celcyclus stoppen om te voorkomen dat replicatie plaatsvindt (met mogelijk de bovengenoemde gevolgen). Bovendien zorgt dit ervoor dat de cel extra tijd heeft om de beschadigingen te repareren. Hiervoor kan de cel een of meerdere van zijn DNA-herstelsytemen gebruiken, die het DNA in zijn normale, onbeschadigde, staat kunnen terugbrengen. Elk herstelsysteem is gespecialiseerd in het herstel van een aantal van de meest voorkomende DNA-lesies (beschadigingen) omdat deze onderling te veel verschillen om door één enkel systeem aangepakt te worden. In sommige gevallen is het DNA dusdanig ernstig beschadigd dat de cel besluit dat een herstelpoging zinloos is; in zulke gevallen kan een cel tot een 'geordende zelfontmanteling' overgaan, een proces dat bekendstaat als apoptose. Door apoptose te plegen voorkomt de cel dat de schade aan zijn DNA het hele organisme schade berokkent (zoals bijvoorbeeld via het ontstaan van kanker). **Hoofdstuk 1** beschrijft kort de reacties van een cel op DNA-schade met de nadruk op de DNA-herstelsystemen.

Het onderzoek in dit proefschrift is toegespitst op één DNA-herstelsysteem: nucleotide excisie herstel (waarvan de engelse afkorting 'NER' is). NER kan een verscheidenheid aan lesies herstellen, waaronder de fotolesies (cyclobutaan pyrimidine dimeren, CPD, en pyrimidine 6-4 pyrimidon fotoproducten, 6-4PP) veroorzaakt door het ultraviolette deel van zonlicht, kleine adducten ontstaan door het antikankermedicijn cisplatina en grote adducten die worden veroorzaakt door de aromatische koolwaterstoffen in verbrand eten en sigarettenrook. NER bestaat uit een aantal stappen. Eerst wordt de beschadiging in het DNA herkend; dan worden de twee DNA-strengen rond de lesie van elkaar gehaald. Daarna wordt de streng waarin de lesie zich bevindt geknipt aan weerszijden van de lesie en het aldus uitgeknipte stuk DNA wordt verwijderd. Als laatste wordt dit verwijderde stuk DNA weer aangemaakt op eenzelfde manier als bij replicatie gebeurt: tegenover de nucleotiden in de overgebleven, onbeschadigde streng worden de juiste nucleotiden geplaatst en het geheel wordt 'aan elkaar geplakt', met als eindresultaat volledig hersteld DNA. NER is uitvoerig onderzocht, en **hoofdstuk 2** geeft een overzicht van de basisconcepten van NER, te beginnen met een korte schets van een van de twee 'onderdelen' van NER, namelijk transcriptie-gekoppeld herstel. Zoals de naam al aangeeft wordt hier herstel van beschadigd DNA gekoppeld aan transcriptie; meer specifiek worden schades waarop een RNA polymerase (eiwit dat transcriptie uitvoert) is vastgelopen sneller hersteld om de blokkering van transcriptie zo snel mogelijk op te heffen. Het andere onderdeel van NER is globaal genoomherstel; dit verwijdert lesies uit het hele genoom en wordt uitgebreid behandeld in hoofdstuk 3. In hoofdstuk 2 worden verder de consequenties besproken van defecten in NER zoals die tot uiting komen in enkele erfelijke ziekten. Patienten met een defect in global genoomherstel lijden aan een ziekte met de naam xeroderma pigmentosum of XP ('perkamenthuid' en pigmentvlekken). Hun hersteldefect uit zich op verschillende manieren maar de meest opvallende (en meest fundamentele) is een extreem verhoogd risico op het ontstaan van huidkanker in delen van de huid die aan de zon blootstaan. Deze eigenschap onderstreept het belang van globaal genoomherstel in het voorkomen van kanker. Aan de andere kant hebben patienten die een defect hebben in transcriptie-gekoppeld herstel geen last van een verhoogde kans op huidkanker. In plaats daarvan hebben ze groeiproblemen en geestelijke handicaps; deze eigenschappen kunnen uiteindelijk worden teruggevoerd op een defect in transcriptie dat wordt beschreven in hoofdstuk 4. Tenslotte beschrijft hoofdstuk 2 twee basisconcepten waar onze huidige manier van denken over NER op is gebaseerd: het idee dat de eiwitten betrokken bij NER een voor een naar de schade worden gehaald (in tegenstelling tot allemaal tegelijk), en het idee dat een DNA-schade niet in één stap wordt herkend maar in twee, waarbij elke stap een andere eigenschap van de schade detecteert.

Zoals hierboven genoemd bevat **hoofdstuk 3** een gedetailleerde weergave van het belangrijkste onderdeel van NER, globaal genoomherstel. De betrokken eiwitten en hun rollen, en hun interacties worden uitvoerig bediscussieerd. Om te beginnen worden de twee zogenaamde 'schadeherkennende factoren', UV-DDB en XPC-hHR23B, behandeld. Terwijl XPC-hHR23B algemeen wordt beschouwd als de factor die globaal genoomherstel begint door beschadigd DNA te herkennen, vervult UV-DDB hier wellicht een ondersteunende rol. De rol van UV-DDB in het herstel van 6-4PP wordt uitvoerig beschreven in hoofdstuk 10. Vervolgens wordt TFIIH behandeld, een complex van meerdere eiwitten dat meerdere functies vervult in de cel. Hoewel eerst werd gedacht dat TFIIH alleen betrokken was bij het openen van de DNA-strengen om andere eiwitten toegang tot het complex te verschaffen, is ondertussen duidelijk dat TFIIH een actieve rol speelt in het reguleren van die eiwitten en misschien zelfs helpt bij het herkennen van de schade. Daarna wordt XPA besproken. Oorspronkelijk werd XPA, samen met RPA, verantwoordelijk gedacht voor het herkennen van schades, maar later bleek dat XPA

in feite betrokken is bij het verzekeren ('verifiëren') van de aanwezigheid van een schade in het DNA. XPA zou dit kunnen doen door de volgende eiwitten die de knippen in het DNA maken te reguleren, mogelijkerwijs samen met TFIIH. Het volgende eiwit, RPA, werd zoals gezegd vroeger betrokken geacht bij schadeherkenning. RPA, dat een buitengewone affiniteit heeft voor enkelstrengs DNA, blijkt echter betrokken bij het stabiliseren van de stukken enkelstrengs DNA die ontstaan na het uit elkaar halen van het DNA rond de schade. Verder is RPA, net als XPA, van belang voor het reguleren van de knip-eiwitten, XPG en ERCC1-XPF. Van XPA, RPA en XPG is niet duidelijk in welke volgorde ze naar het NER-complex komen. De bewijzen voor en tegen verschillende bindingsvolgorden worden besproken, alsmede het voorstel dat deze drie eiwitten niet in een vaste volgorde binden, maar willekeurig. ERCC1-XPF is het laatste eiwit dat in het NER-complex wordt opgenomen voordat de 'dubbele knip' plaatsvindt die het beschadigde stuk DNA verwijdert. Hierna worden alle eiwitten die betrokken zijn bij de eerste stappen van NER verwijderd van het DNA; alleen RPA en mogelijk XPG blijfven gebonden (zie ook hoofdstuk 10). Vervolgens worden eiwitten aangetrokken die ook betrokken zijn bij replicatie. PCNA, dat een ring vormt die om het DNA bindt, en waaraan de DNA polymerases binden; RF-C, het eiwit dat PCNA om het DNA helpt; en DNA polymerases delta en/of epsilon. Beide polymerases kunnen deze stap van NER uitvoeren; zie ook hoofdstuk 10. Tenslotte wordt DNA ligase I gerecruteerd, een eiwit dat het nieuw aangemaakte stuk DNA 'vastplakt' aan het oude stuk DNA.

Er bestaat nog een koppeling tussen transcriptie en DNA-herstel die nog niet is genoemd. Na het toebrengen van DNA-schades die door transcriptie-gekoppeld herstel worden behandeld (zoals de al genoemde door UV-straling veroorzaakte fotolesies) wordt transcriptie geremd. Deze remming wordt normaal gesproken weer opgeheven naarmate het DNA wordt gerepareerd. Opvallend genoeg wordt transcriptie ook afgeremd in genen die geen lesies bevatten, en de oorzaak hiervan is nog onbekend. In **hoofdstuk 4** worden de eigenschappen en enkele mogelijke oorzaken voor dit fenomeen besproken. Het aanbrengen of verwijderen van fosfaat (een verbinding van één atoom fosfor en vier atomen zuurstof: PO_4) aan het RNA polymerase bljikt hier een belangrijke rol te spelen: in de aanwezigheid van DNA-schade wordt fosfaat aan het RNA polymerase aangebracht. Dit zorgt ervoor dat het polymerase niet meer begint aan de transcriptie van genen; hierdoor wordt de transcriptie geremd van alle genen, ook als ze niet beschadigd zijn. In normale cellen wordt dit fosfaat weer verwijderd als de DNA-schades zijn gerepareerd; in cellen van CS-patienten echter is dit niet het geval. De permanente remming van transcriptie die zo ontstaat ligt waarschijnlijk ten grondslag aan veel van de symptomen van CS-patienten.

In het laatste inleidende hoofdstuk, **hoofdstuk 5**, wordt NER in de context van de celkern geplaatst. Het DNA van de cel bevindt zich in de kern, maar er is meer in de kern dan alleen DNA. De belangrijkste andere component in de kern zijn eiwitten die het DNA heel compact ingepakt houden; deze combinatie van DNA en eiwitten heet 'chromatine' en heeft in principe een negatieve invloed op NER (omdat het minder goed bij het DNA kan, ingepakt als het is door de andere eiwitten). Er bestaan echter een aantal mechanismes die NER helpen door het chromatine te openen, waardoor NER beter bij het DNA kan en het herstel ervan gestimuleerd wordt. Deze mechnismen en enkele van de eiwitten die erin een rol spelen worden besproken. Nadat NER zijn werk heeft gedaan wordt de oorspronkelijke structuur van het chromatine hersteld; de eiwitten die hiervoor zorgen worden ook besproken.

Het meeste onderzoek naar NER is gedaan '*in vitro*', d.w.z. met extracten van cellen en/of met gezuiverde eiwitten. Het onderzoek in dit proefschrift echter is voornamelijk uitgevoerd in intacte cellen. Het grote voordeel hiervan is dat de omstandigheden waaronder een experiment wordt uitgevoerd (bijvoorbeeld de zoutconcentratie of de activiteit van gezuiverde eiwitten) geen invloed kunnen uitoefenen op de uitkomst ervan. De meest gebruikte techniek in dit proefschrift is de zogenaamde 'immunofluorescentie'. Hierbij worden cellen eerst met een chemische stof 'gefixeerd', als het ware chemisch bevroren. Daarna worden antilichamen (vandaar 'immuno') in de cellen gebracht die niet bedoeld zijn om ziekteverwekkers op te sporen, maar díe eiwitten waarin de onderzoeker geïnteresseerd is – in dit geval dus NER-eiwitten. Deze antilichamen kunnen op hun beurt voorzien worden van een lichtgevend label (vandaar 'fluorescentie'). Dit label kun je zien als de cel onder de microscoop wordt gelegd en zo kan het gedrag van de eiwitten worden bestudeerd.

In **hoofdstuk 6** is immunofluorescentie gebruikt om de volgorde te bepalen waarin verschillende NER-eiwitten naar de schade toegaan. Om dit mogelijk te maken hebben we een methode ontwikkeld om NER en andere processen die met UV-schades te maken hebben te bestuderen. Dit is een elegante en eenvoudige methode waarin de cellen, die groeien op een klein dun glaasje, worden bedekt met een dun filter van polycarbonaat (dat normaal wordt gebruikt om oplossingen te filteren) met kleine gaatjes die een klein deel van de cel, en dus ook de kern, onbedekt laten. Als de cellen met UV worden bestraald, blokkeert het polycarbonaat het UV zodat de celkernen (en dus het DNA) alleen aan UV worden blootgesteld direct onder de gaatjes. Deze methode wordt daarom meestal 'lokale bestraling' genoemd; een schematische weergave ervan is te vinden in hoofdstuk 8, figuur 5. Processen die reageren op de UV-lesies vinden nu specifiek plaats in deze 'spots' en kunnen zichtbaar gemaakt worden met bijvoorbeeld immunofluorescentie. XPC bleek naar DNA-schades te gaan in cellen waarin XPA niet aanwezig was (deze cellen waren afkomstig van XP-patienten), maar vice versa ging XPA niet naar schades als XPC niet aanwezig was: XPC bevindt zich dus vóór XPA in globaal genoomherstel. Op een soortgelijke manier werd vastgesteld dat XPA en XPG elkaar niet beïnvloedden, terwijl we tenslotte vonden dat ERCC1-XPF zich na XPA naar de schade begaf.

Hoofdstuk 7 beschrijft in meer detail de bovenstaande methode van 'lokale bestraling'. In dit hoofdstuk is de methode verder gebruikt om de relatie te bestuderen tussen UV-bestraling en de bovengenoemde remming van transcriptie. We zagen dat de remming beperkt bleef tot die delen van de celkern die UV-lesies bevatten, terwijl in de rest van de kern transcriptie op het normale niveau bleef. Onze conclusie was dat het signaal voor het reduceren van transcriptie niet werd overgebracht door een factor die vrij door de hele kern kan bewegen, zoals TFIIH (dat een kandidaat was voor deze rol).

Een techniek die veel gebruikt wordt in het onderzoek van levende cellen is om de eiwitten waarin men geïnteresseerd is te koppelen aan het zgn. groen fluorescerend eiwit (GFP is de engelse afkorting hiervan). Dit zorgt ervoor dat dit eiwit gevolgd kan worden in levende cellen met behulp van een gevoelige fluorescentiemicroscoop – de cellen hoeven dus niet zoals bij immunofluorescentie eerst gefixeerd te worden. In **hoofdstuk 8** wordt het gedrag van een specifiek aan GFP gekoppeld NER-eiwit, XPA, beschreven. Door zijn diffusiesnelheid – de snelheid waarmee het door de cel beweegt – te meten kon worden bepaald dat XPA door de kern bewoog zonder zich in een groot eiwitcomplex te bevinden (zoals bijv. een 'repairosoom', d.w.z. een complex van alle NER-eiwitten). Dit was extra bewijs voor het model dat is beschreven in hoofdstuk 2, waarin de NER-eiwitten zich een voor een naar de lesie begeven. Ook vonden we dat na de 'lokale' UV-bestraling RPA in het NER-complex kon worden opgenomen in afwezigheid

van XPA, wat een aantal oudere publicaties weerlegde, die claimden dat XPA zich in een complex bevond met RPA.

Het eiwit UV-DDB lijkt slechts noodzakelijk te zijn voor het herstel van één type DNA-lesie, de CPD; alle andere lesies kunnen onafhankelijk van UV-DDB worden hersteld na herkenning door XPC-hHR23B. Ook het herstel van 6-4PP leek lange tijd niet beinvloed te worden door de aan- of afwezigheid van UV-DDB. Dit was raadselachtig, omdat UV-DDB een hogere affiniteit voor 6-4PP heeft dan voor CPD – de affiniteit waarmee een schade-herkennend eiwit aan een lesie bindt hangt meestal samen met de snelheid van herstel ervan. Zoals beschreven in **hoofdstuk 9** konden we middels het gebruik van een aantal methoden laten zien dat UV-DDB wel degelijk een grote invloed kan hebben op het herstel van 6-4PP Omdat UV-DDB de opmerkelijke eigenschap heeft te worden afgebroken tijdens herstel was het effect echter beperkt tot een klein aantal lesies. Als een kleine hoeveelheid 6-4PP werd geïnduceerd werd hun herstel sterk versneld door de aanwezigheid van UV-DDB; boven een bepaalde 'drempel' echter verdween dit effect. In dat geval werd herstel onafhankelijk van UV-DDB voortgezet omdat zoals gezegd 6-4PP, in tegenstelling tot CPD, door XPC kunnen worden herkend en vervolgens hersteld.

Volgens *in vitro* onderzoek zijn de DNA polymerases delta en epsilon en DNA ligase I betrokken bij de laatste twee stappen van NER: het synthetiseren van DNA in het gat dat overblijft na het verwijderen van het beschadigde stuk DNA, en het vastmaken van het nieuwe aan het oude stuk DNA. In **hoofdstuk 10** presenteren we, na gebruik te hebben gemaakt van de lokale bestraling en immunofluorescentie, sterke aanwijzingen dat dit inderdaad het geval is *in vivo*, omdat zowel DNA polymerases delta als epsilon en DNA ligase I zich ophoopten op de plaatsen van UV-schade. Door cellen twee keer met UV te bestralen, een keer de gehele cel en een keer 'lokale' bestraling, konden we bepalen of eiwitten gebonden bleven aan het NER-complex op de lesie of niet. We vonden onder andere dat alle eiwitten die betrokken zijn bij de stappen in NER tot en met de dubbele knip, na deze knippen uit het complex verdwenen voordat de volgende eiwitten (PCNA, RF-C, enz.) werden aangetrokken. De enige uitzondering hierop was RPA, dat wel gebonden bleef, waarschijnlijk om het stuk enkelstrengs DNA te beschermen en tegelijk te helpen PCNA aan te trekken. Aan de andere kant vonden we dat als de dubbele knippen niet konden plaatsvinden de eiwitten stevig aan het complex gebonden bleven, en dat de eiwitten betrokken bij de laatste stappen van NER niet naar het complex gingen.

Abbreviations

6-4PP	pyrimidine (6-4) pyrimidone photoproduct(s)
8-oxoguanine	7,8-dihydro-8-oxoguanine
ACF	ATP-utilising chromatin assembly and remodelling factor
ARR	access, repair, restore
Asf1	anti-silencing function 1
ATP	adenosine triphosphate
BER	base excision repair
bp	base pair(s)
CAF-1	chromatin assembly factor 1
CAK	CDK (cyclin-dependent kinase) activating kinase
CBP	CREB (cAMP-responsive element binding protein) binding protein
cisplatin	*cis*-diamminedichloroplatinum(II)
COP9	constitutively photomorphogenic 9
CPD	cyclobutane pyrimidine dimer
CS	Cockayne syndrome
DDB	damaged DNA binding
DNA	deoxyribonucleic acid
DNA-PKcs	DNA protein kinase catalytic subunit
DSBR	double strand break repair
dsDNA	double strand DNA
FEN	flap endonuclease
gadd	growth arrest after DNA damage
GFP	green fluorescent protein
GGR	global genome nucleotide excision repair
(h)HR23	(human) homolog of *S. cerevisiae* Rad23
hMLH	human homolog of *E. coli* MutL
HMGN	high mobility group N
hMSH	human homolog of *E. coli* MutS
ISWI	imitation SWI
MEFs	Mouse embryonic fibroblasts
MGMT	methylguanine DNA methyltransferase
NA-AAF	N-acetoxy-2-acetylaminofluorene
NER	nucleotide excision repair
nt	nucleotide(s)
PCNA	proliferating cell nuclear antigen
PH	pleckstrin homology
PMS	postmeiotic segregation
pol	DNA polymerase
RF-C	replication factor C
RNA	ribonucleic acid
RNA pol	RNA polymerase
RPA	replication protein A

RRS	recovery of RNA synthesis
ssDNA	single strand DNA
STAGA	SPT3-TAF(II)31-GCN5L acetylase
SUMO	small ubiquitin-like modifier
SWI/SNF	(activation of) mating switch and sucrose non-fermenting (genes)
TCR	transcription-coupled nucleotide excision repair
TFIIH	transcription (initiation) factor IIH
TFTC	TBP-free TAF(II)-containing
thymine glycol	5,6-dihydro-5,6-dihydroxythymine
TTD	trichothiodystrophy
UDS	unscheduled DNA synthesis
UV	ultraviolet light
UVB	medium wavelength component of ultraviolet (UV) light
UVC	shortwave component of ultraviolet (UV) light
UV-DDB	UV-damaged DNA binding protein
XP	xeroderma pigmentosum

Curriculum vitae

Naam	Marcel Volker
Geboren	18 januari 1976 te Leiderdorp
september 1988 – juni 1994	VWO aan het Pieter Groen College (later Andreas College geheten) te Katwijk
september 1994 – maart 1999	Scheikunde aan de Rijksuniversiteit Leiden; theoretische specialisaties organische chemie en moleculaire biologie/biochemie
september 1997 – januari 1999	Hoofdvakstage aan de afdeling Moleculaire Genetica o.l.v. Dr. J. Brouwer: Nucleotide excisie herstel in S. cerevisiae
februari 1999 – augustus 2003	Promotieonderzoek aan de afdeling Stralengenetica en Chemische Mutagenese (later Toxicogenetica geheten) o.l.v. promotores Prof. Dr. Ir. A.A. van Zeeland en Prof. Dr. L.H.F. Mullenders: Interplay between transcription and NER in intact human cells.
april 2004 – heden	Post-doctoral research fellow in het laboratorium van Prof. Dr. A.R. Lehmann, Brighton, Groot-Brittannië: genotype-phenotype relationship in XP-D and XP-D/CS cells

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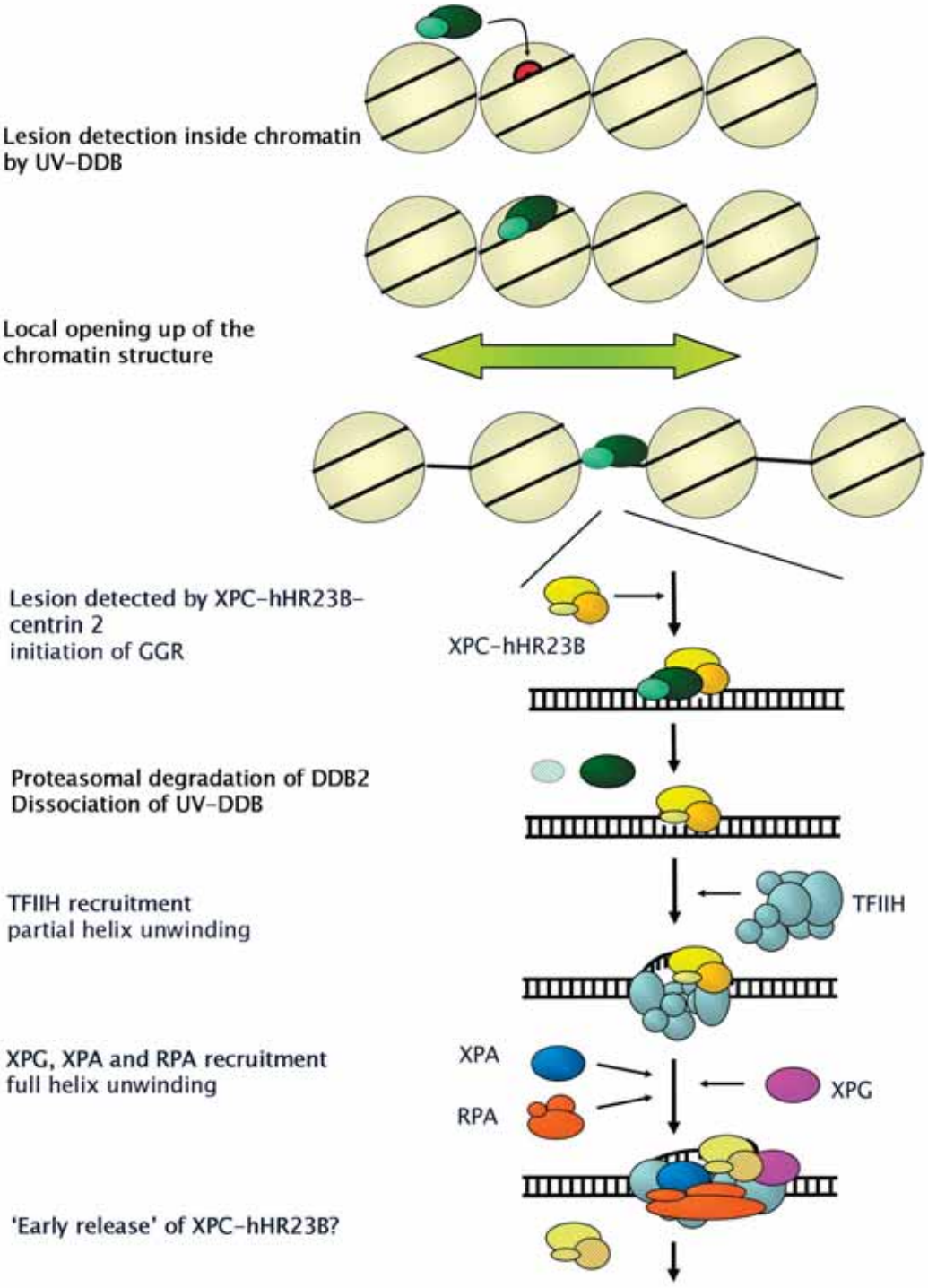
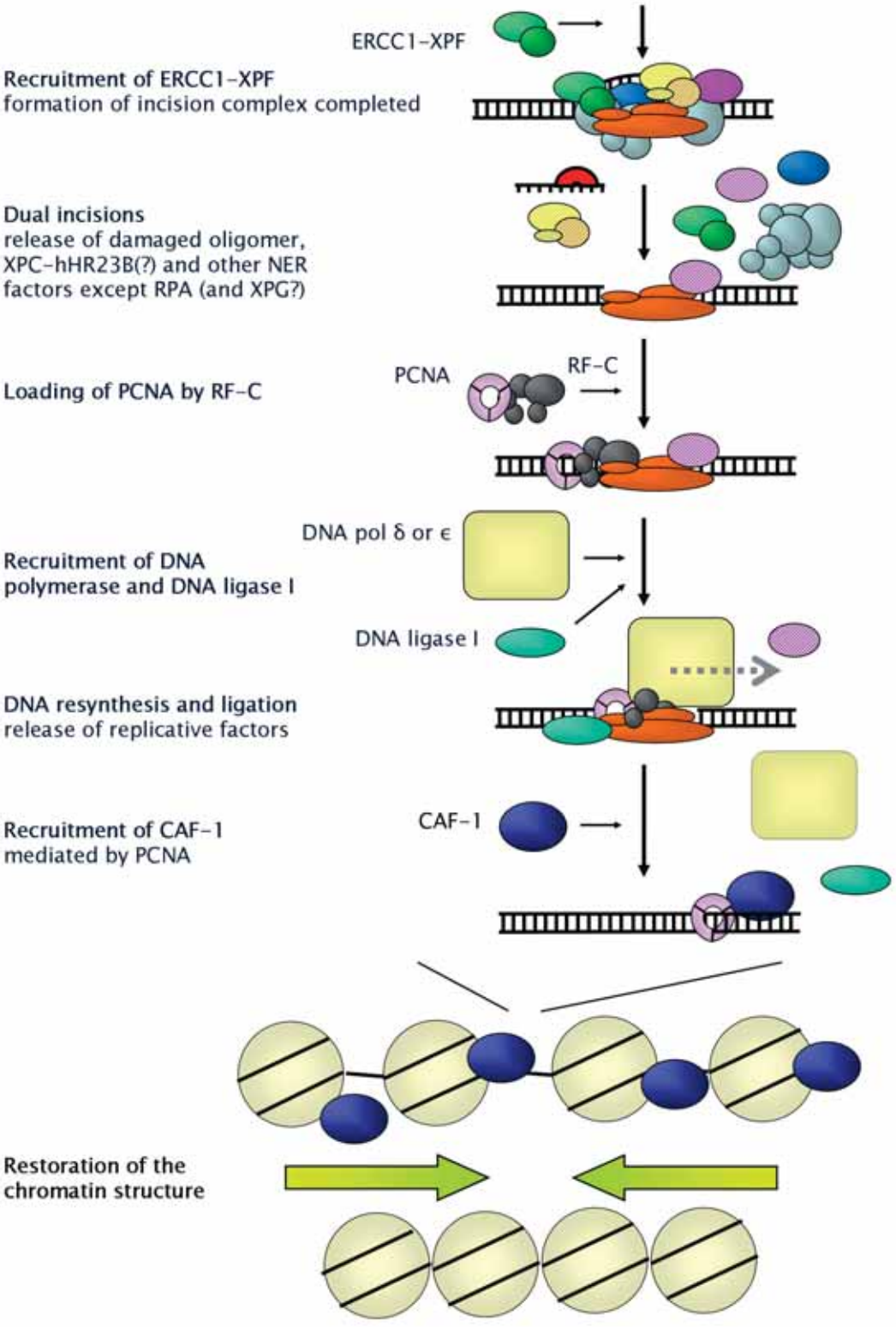
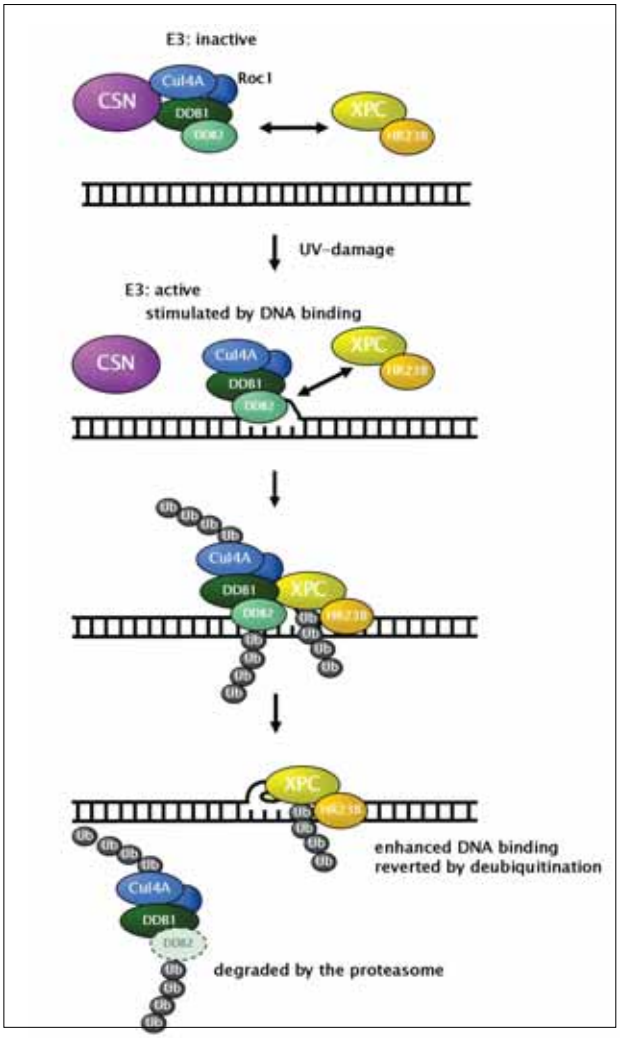
Appendix
Selected colour figures

Global genome repair in chromatin

Access, repair, restore




ERCC1-XPF
Recruitment of ERCC1-XPF
formation of incision complex completed
Dual incisions
release of damaged oligomer,
XPC-hHR23B(?) and other NER
factors except RPA (and XPG?)
PCNA
RF-C
Loading of PCNA by RF-C
DNA pol δ or ϵ
Recruitment of DNA
polymerase and DNA ligase I
DNA ligase I
DNA resynthesis and ligation
release of replicative factors
CAF-1
Recruitment of CAF-1
mediated by PCNA
Restoration of the
chromatin structure



Chapter 3, Figure 2. UV-induced UV-DDB-dependent ubiquitination of XPC

In unirradiated cells, the UV-DDB-associated E3 is inactivated by its interaction with the COP9 signalosome (CSN). Thus, XPC is not ubiquitinated, despite its interaction with UV-DDB. Upon UV irradiation, UV-DDB binds to lesions, particularly 6-4PP. CSN dissociatiates from E3, activating it.

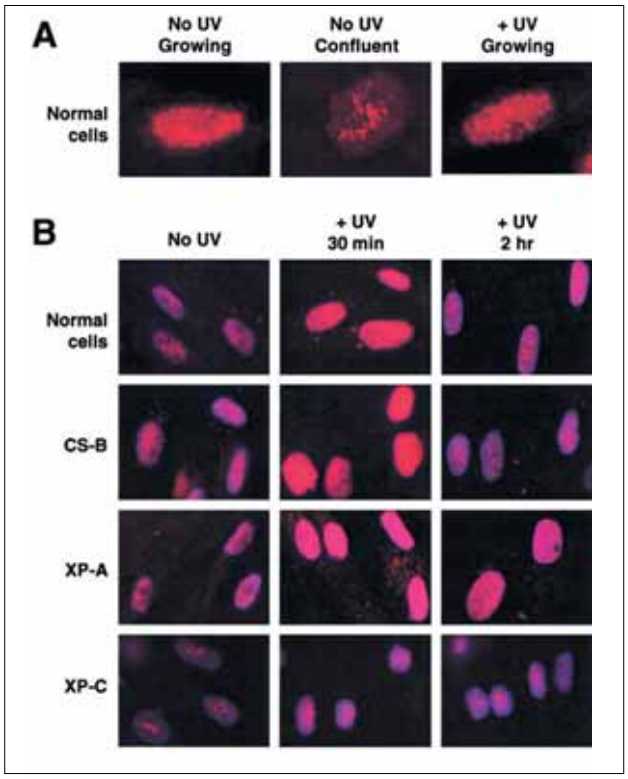
The binding of UV-DDB to the lesion further stimulates E3 activity. The activated UV-DDB-E3 then recruits XPC, and XPC and DDB2 are ubiquitinated at the lesion site. Polyubiquitinated UV-DDB loses its damaged DNA binding activity, whereas the DNA binding of XPC is enhanced by its ubiquitination. This results in the displacement of UV-DDB by XPC on the lesion. Ubiquitinated DDB2 is degraded by the proteasome. The ubiquitinated form of XPC reverts to the unmodified form through deubiquitation. Adapted from Sugasawa et al., 2005.

Previous pages: Chapter 3, Figure 1. Global genome repair inside chromatin – access, repair, restore

Schematic depiction of GGR placed within the context of chromatin. Upon lesion recognition (at least for UV lesions this is likely to involve UV-DDB, but other lesions may also be recognised by UV-DDB), chromatin remodelling enzymes are recruited to the lesion site. Local opening of the chromatin structure provides access for the core GGR machinery, and the lesion is bound by the essential damage recognition factor XPC-hHR23B (which in vivo is associated with centrin 2). XPC-hHR23B(-centrin 2) slightly opens up the DNA around the lesion and recruits TFIIH. This factor uses its two helicases, XPB and XPD, to fully open up the helix, and subsequently XPA, RPA and XPG are recruited. RPA binds to the single-strand DNA formed by the consecutive actions of XPC-hHR23B(-centrin 2) and TFIIH; XPA is the damage verification protein and XPG is one of the two structure-specific endonucleases employed by NER. Some reports suggest that at this stage, XPC-hHR23B (-centrin 2) is released from the complex (Wakasugi and Sancar, 1998; Riedl et al., 2003); however in our experiments we were unable to provide support for this notion (chapter 10). The recruitment of XPA is a prerequisite for the incorporation of the second structure-specific endonuclease, ERCC1-XPF into the complex, completing the formation of the preincision complex. The next step in the repair reaction is dual incision, followed by the release of several, but not all, repair factors. RPA remains bound to the single-strand DNA, as may XPG (which contains a PCNA-binding motif). The latter stages of repair are carried out by proteins also functioning during DNA replication: RF-C loads PCNA onto the DNA, DNA polymerases δ or ε can synthesise across the gap, and DNA ligase I is able to seal the remaining nick. Post-repair restoration of the chromatin structure involves CAF1, recruited to the site of repair by PCNA which has remained bound to the DNA. See text for details.

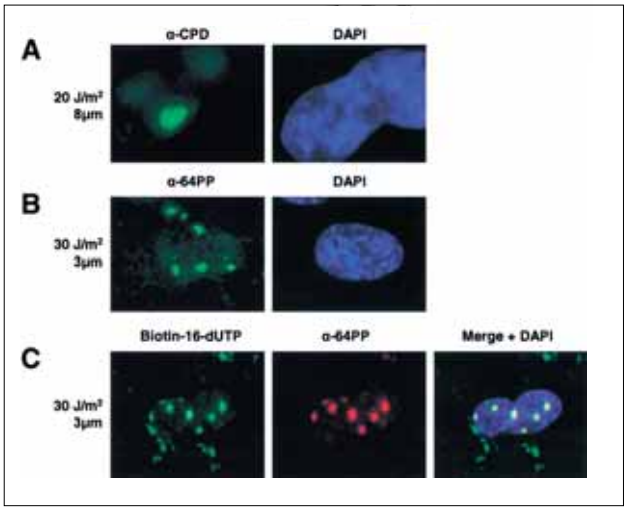
Chapter 6, Figure 1. Altered Nuclear Distribution of TFIIH in Confluent Human Fibroblasts after Global UV Irradiation

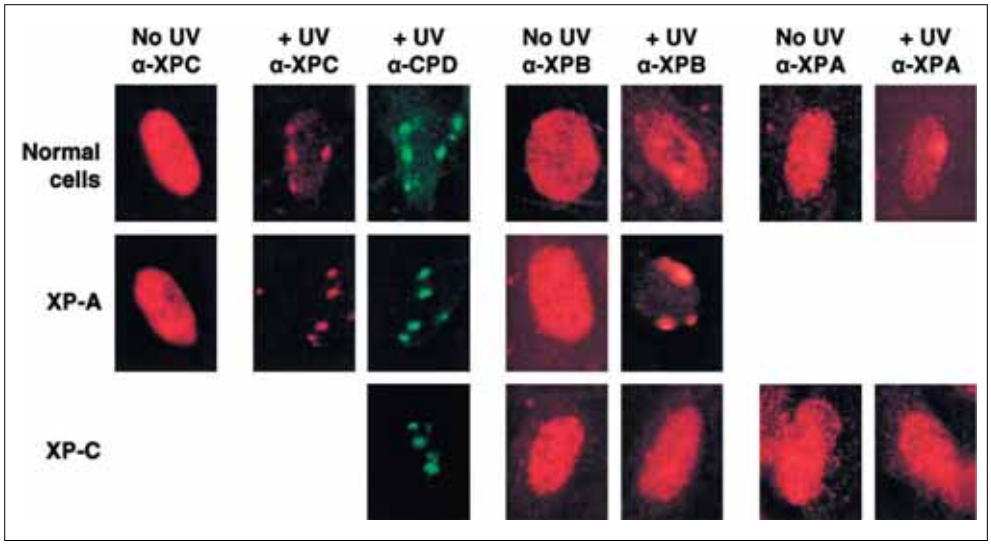
(A) Growing or confluent normal human fibroblasts (VH25) were fixed and immunolabeled employing an antibody against XPB or immunolabeled at 30 min after UV irradiation (10 J/m^2). (B) Confluent normal human (VH25), CS-B (CS1AN), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were either fixed and immunolabeled using an antibody against XPB or exposed to UV (10 J/m^2) and immunolabeled 30 min or 2 hr later.



Chapter 6, Figure 3. UV Exposure through Isopore Polycarbonate Filters Causes Locally Damaged Areas in the Nuclei

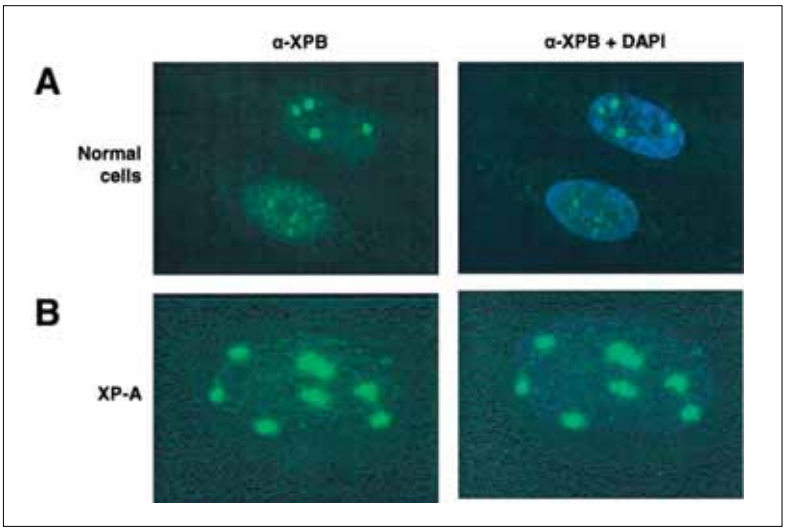
Normal human fibroblasts (VH25) were UV-irradiated with 20 or 30 J/m^2 through a 3 or 8 µm pore filter and immediately fixed. Immunofluorescent labeling was performed using (A) an antibody against CPD (α-CPD) or (B) an antibody against 6-4PP (α-64PP). In addition (C), VH25 cells were locally exposed to UV radiation, incubated for 20 min in culture medium, and permeabilized, after which run-on DNA synthesis in the presence of biotin-dUTP was carried out. Cells were subsequently immunolabeled for both DNA repair synthesis (biotin-16-dUTP) and the presence of DNA damage, i.e., 6-4PP (α-64PP).





Chapter 6, Figure 4. Recruitment of NER Proteins to Sites of UV Damage

Normal human (VH25), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were UV exposed to 30 J/m^2 through 3 µm filters or mock irradiated and at 15 min after exposure immunolabeled with an antibody (red) against XPC and an antibody (green) against CPD or labeled with antibodies against XPB or XPA.

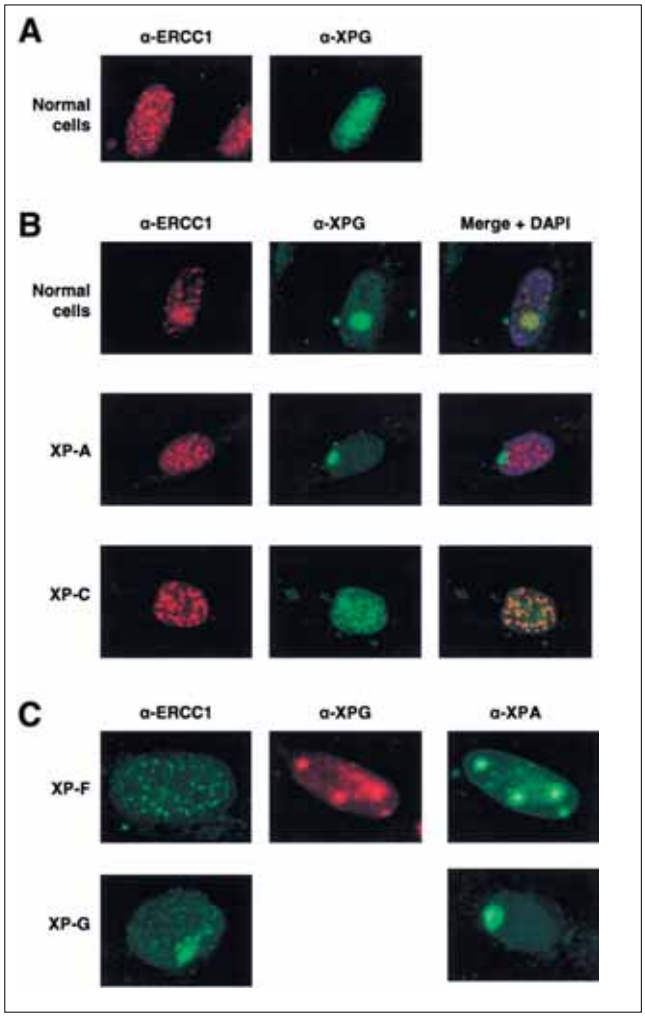


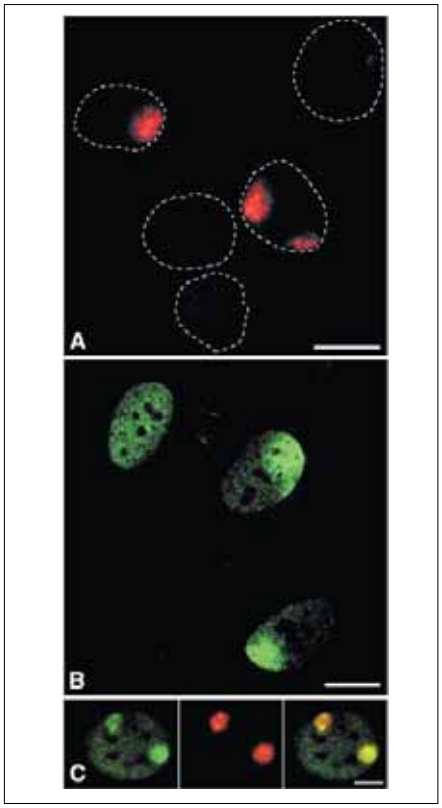
Chapter 6, Figure 5. Reduction of XPB in Unexposed Parts of the Nucleus after Local UV Irradiation

Normal human fibroblasts (VH25) (A) or XP-A cells (XP25RO) (B) were UV irradiated with 30 J/m^2 through 3 µm pore filters and immunolabeled for XPB 15 min or 4 hr after UV exposure, respectively.

Chapter 6, Figure 6. Recruitment of the NER Endonucleases to the Sites of UV Damage

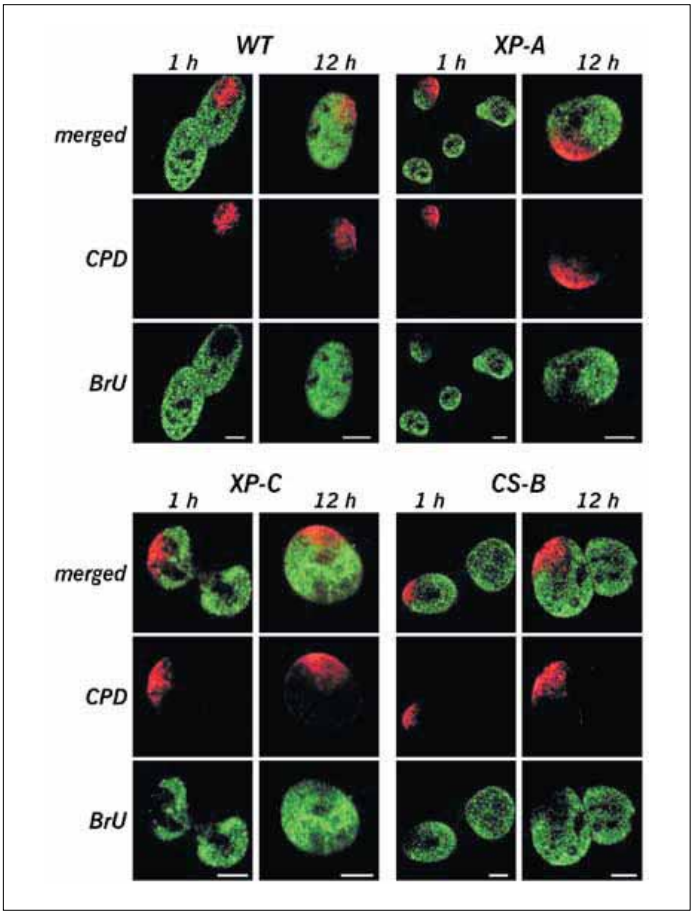
Confluent fibroblasts were UV irradiated (30 J/m^2) through 3 µm filters or mock irradiated, fixed 15 min after UV exposure and immunolabeled for ERCC1, XPG, or XPA. (A) Unexposed normal human fibroblasts (VH25) were immunolabeled for ERCC1 and XPG. (B) Normal human (VH25), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were immunolabeled for ERCC1 and XPG. (C) XP-F (XP24KY) and XP-G (XPCS1RO) fibroblasts were immunolabeled for XPG and ERCC1, respectively, or XPA.





Chapter 7, Figure 1

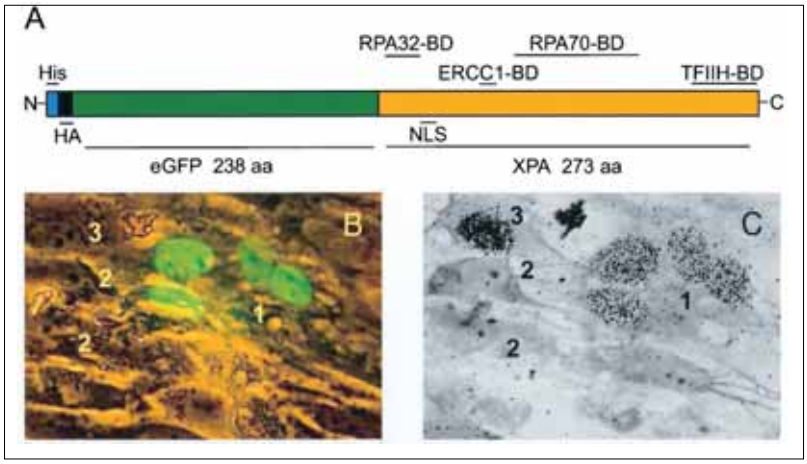
(A) Detection of locally induced UV damage in cell nuclei. A UV-blocking polycarbonate filter containing pores of 5 µm in diameter was used to cover a monolayer of cells. The filter-covered cells were UV-irradiated with 30 J/m^2 and CPDs were subsequently detected by immunofluorescent labelling. Dotted lines denote the contours of individual cell nuclei. Two nuclei show labelling of CPDs in discrete nuclear areas. (B) Effect of local nuclear UV damage on the distribution of TFIIH. Human primary fibroblasts were locally UV-irradiated with 100 J/m^2 UV light, using a filter with 10 µm pores. Following irradiation, cells were grown for 1 h and immunolabelled against the p62 subunit of TFIIH. The top-left nucleus displays the characteristic labelling pattern of TFIIH in unirradiated cells, whereas the two remaining nuclei exhibit a TFIIH accumulation in UV-damaged nuclear areas, and a reduction in TFIIH signal outside these areas. A single confocal optical section is shown. (C) Colocalization of TFIIH (green) and CPDs (red). Human primary fibroblasts were locally irradiated with 30 J/m2 UV light, using a filter with 8 µm pores. Following irradiation, cells were grown for 30 min and dual labelled against both CPDs and the p62 subunit of TFIIH. Bars represent 10 µm.



Chapter 7, Figure 2. Effect of local nuclear UV damage on transcription

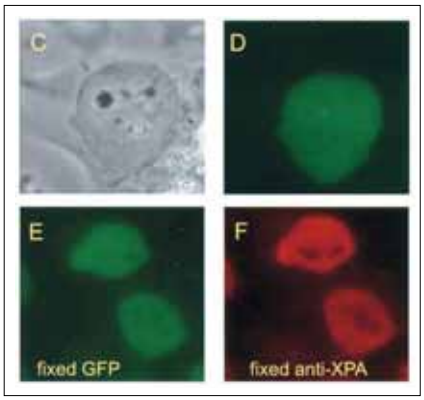
Normal human primary fibroblasts (WT; NER-proficient) and immortalized primary fibroblasts from patients suffering from xeroderma pigmentosum group A (XP-A; no NER), group C (XP-C; no GGR) or Cockayne syndrome group B (CS-B; no TCR) were studied.

Exponentially growing cells were locally UV-irradiated with 50 J/m^2 using filters containing 10 µm pores. After irradiation, cells were cultured for either 1 or 12 h. Subsequently, cells were allowed to incorporate BrUTP into nascent RNA during run-on transcription labelling. Nascent RNA (green) and CPDs (red) were dual labelled by immunostaining. Single confocal optical sections are shown. Bars represent 5 µm.



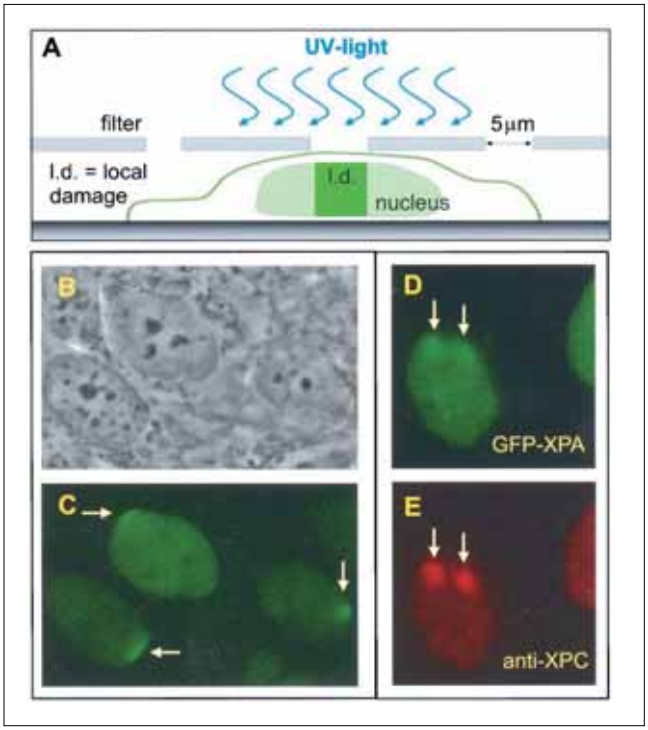
Chapter 8, Figure 1. Functionality of GFP-XPA

(A) Schematic representation of the His9-HA-eGFP–XPA fusion gene with the different binding domains indicated. NLS, nuclear localization signal; BD, binding domain; aa, amino acids. (B) Fluorescence image of XP-A cells injected with GFP-tagged XPA cDNA. Only the multinucleated cell microinjected with GFP-XPA cDNA showed a homogeneous nuclear expression (number 1); surrounding cells were not injected (number 2). (C) Measurement of the repair capacity of cells with fluorescent nuclei by means of UV-induced UDS (see Materials and Methods). The amount of silver grains above the nuclei of the injected cells (number 1) was comparable to what was seen with wild-type cells (not shown), whereas the surrounding XP-A fibroblasts (number 2) show the low level of DNA synthesis typical for UV-exposed XP-A cells. The cell indicated with the number 3 is in S phase.



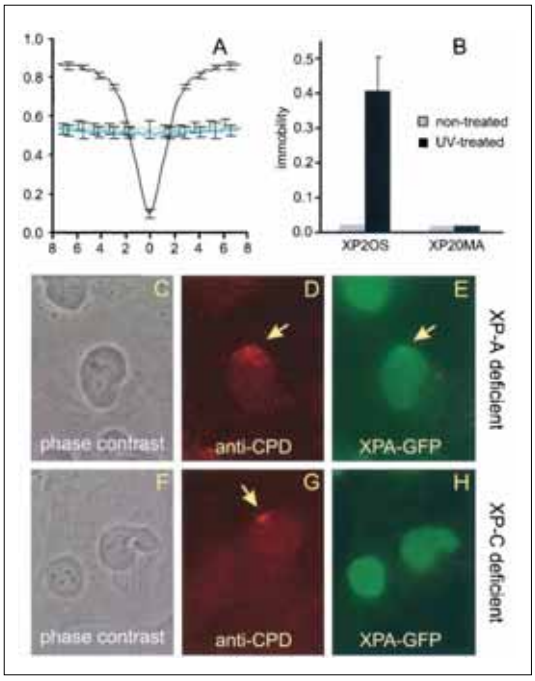
Chapter 8, Figure 2. Expression and characterization of XP2OS cells stably expressing GFP-tagged XPA

(C) Phase-contrast image of a living GFP-XPA-transfected XP2OS, clone 40 cell. (D) Epifluorescence GFP image of the same cell as in panel C, showing a homogeneous nuclear distribution. (E) Fluorescence image after fixation of clone 40, showing a similar distribution as in panel D. (F) Immunofluorescence of the same cell as in panel E incubated with anti-XPA serum, showing a similar XPA distribution as with GFP fluorescence, except for the nucleoli.



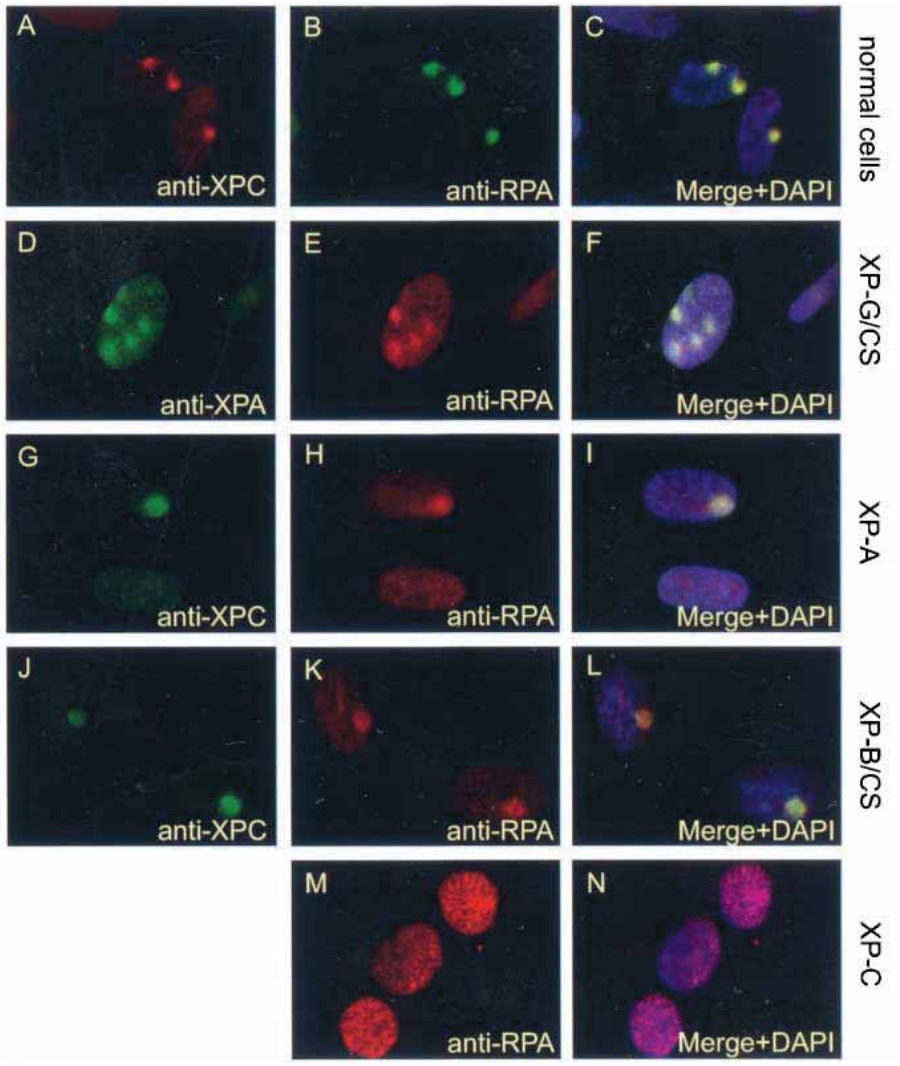
Chapter 8, Figure 5. Accumulation of GFP-XPA within restricted nuclear areas after local UV irradiation

(A) Schematic presentation of local UV damage infliction on living cultured cells. (B and C) Micrographs, phase-contrast image [B] and fluorescence image [C] of living cells expressing GFP-XPA (clone 40) and UV-irradiated through a filter with small (5 μm-diameter) pores. The arrows in panel C point to the local accumulations of GFP-XPA. (D and E) GFP-XPA accumulations (arrows) shown in panel D clearly colocalize with endogenous XPC (E) concentrations, as determined with anti-XPC antibodies, in fixed cells.



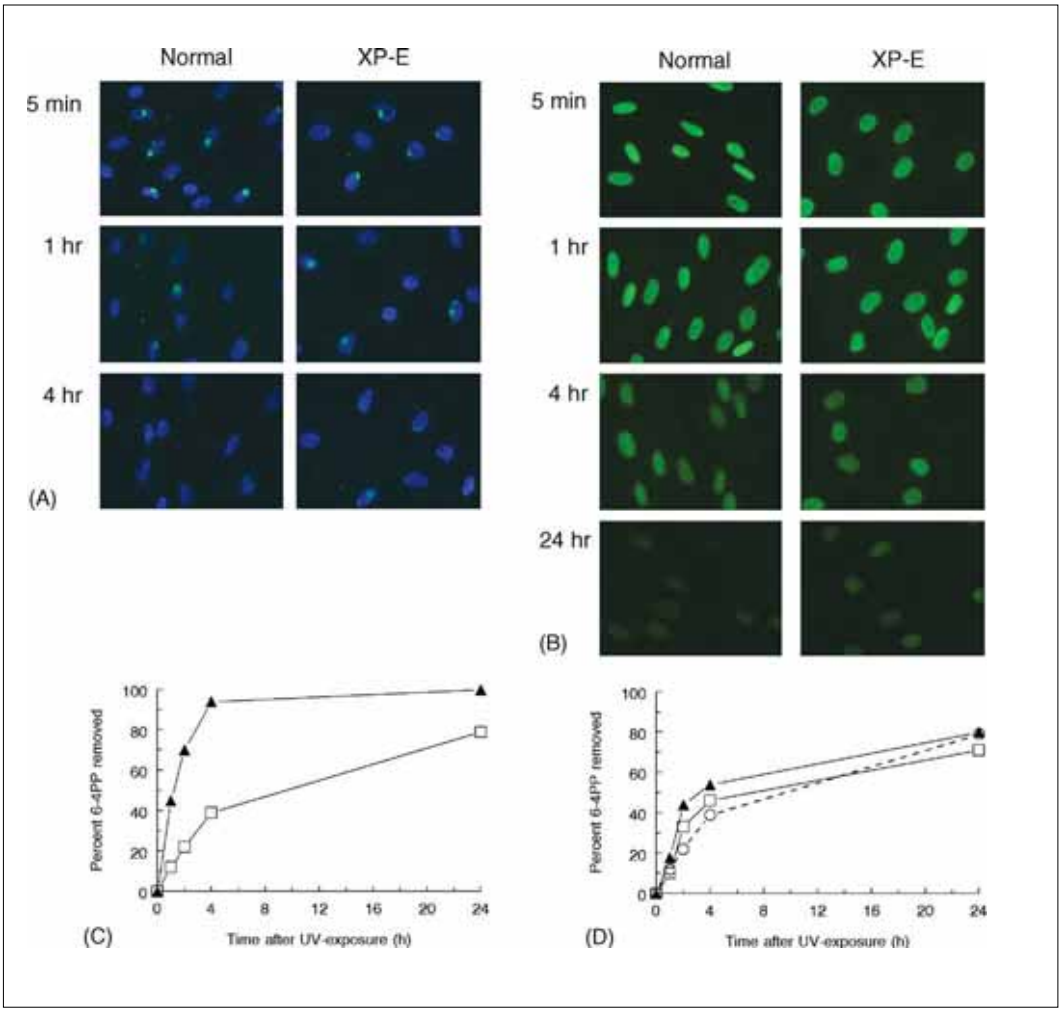
Chapter 8, Figure 7. Effect of XPC on damage-induced XPA immobility as analyzed by FRAP-FIM and local damage induction

(A) FRAP-FIM profile of GFP-XPA expression in XP20MA (XP-C) cells. Shown are results for nonirradiated cells (light green line), cells irradiated at 16 J/m^2 (blue line), and fixed cells (black line). UV-exposed XP20MA cells do not show any GFP-XPA immobilization. (B) Quantification of immobilization of GFP-XPA in XP20MA and XP2OS cells with and without UV irradiation. (C) Phase-contrast image of GFP-XPA-expressing XP2OS cells. (D) Anti-CPD immunostaining in a GFP-XPA-expressing XP2OS cell. The arrow indicates the site of the damage. (E) GFP image of the same cell as in panel D, showing enrichment of GFP-XPA at the damaged site. (F) Phase-contrast image of GFP-XPA-expressing XP20MA cells. (G) Anti-CPD immunostaining of a GFP-XPA-expressing XP20MA cell, indicated by the arrow. (H) GFP image showing no enrichment of GFP-XPA molecules.



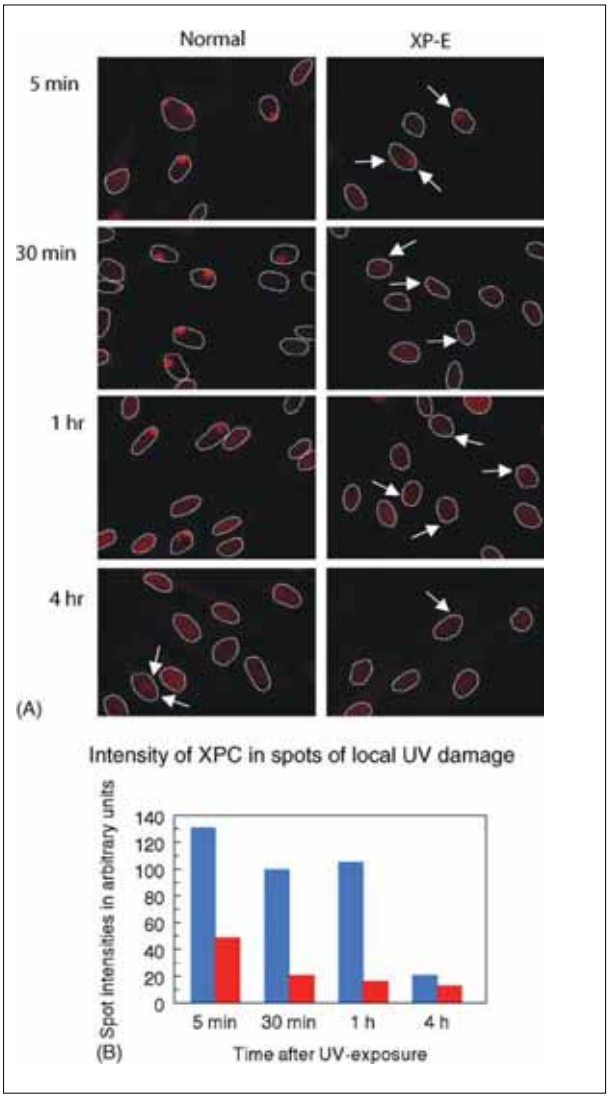
Chapter 8, Figure 8. Relocalization of RPA to the NER complex 30 min after local irradiation with 25 J/m² UV

(A to C) Anti-XPC (A) and anti-RPA (B) immunostaining of VH25 cells and the merged image after immunostainings plus DAPI nuclear DNA staining (C). (D to F) Anti-XPA (D) and anti-RPA (E) immunostaining of XPCS1RO cells and the merged image after immunostainings plus DAPI nuclear DNA staining (F). (G to I) Anti-XPC, G and anti-RPA (H) immunostaining of XP25RO cells and the merged image after immunostainings plus DAPI nuclear DNA staining. (J to L) Anti-XPC (J) and anti-RPA (K) staining of XP131MA cells and the merged image after immunostainings plus DAPI nuclear DNA staining (L). (M and N) Anti-RPA immunostaining of XP21RO cells (M) and the merged image after immunostaining plus DAPI nuclear DNA staining (N). The yellow color in the merged images in panels C, F, I, and L indicates colocalization of NER proteins at sites of locally induced DNA damages.



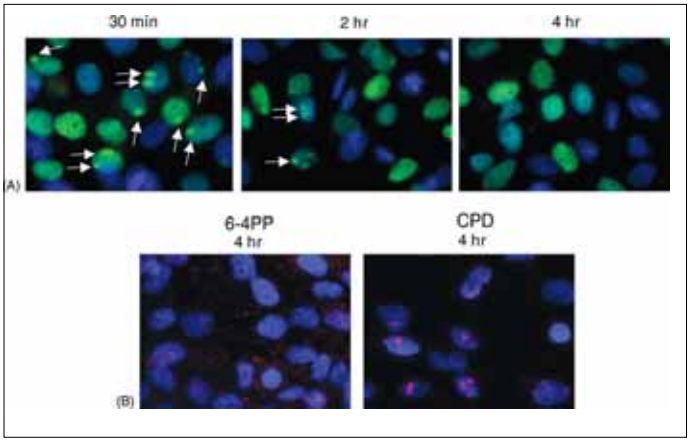
Chapter 9, Figure 3. Accelerated 6-4PP repair in normal human fibroblasts (VH10hTert) compared to XP-E fibroblasts (GM01389hTert) after local UV irradiation, but not after global UV irradiation as measured by immunofluorescence

(A) Fluorescent immunostaining of 6-4PP (green) at various times after local UV irradiation (30 J/m^2) through an 8 µm pore size filter. Images were taken with equal exposure times and merged with DAPI nuclear DNA staining (blue). (B) Fluorescent immunostaining of 6-4PP (green) at various times after global UV irradiation (30 J/m^2). Images were taken with equal exposure times. (C and D) Graphs presenting the percentage of 6-4PP removed determined from multiple (>20) fluorescent images for each timepoint. (C) Removal of 6-4PP in (▲) normal human and (□) XP-E cells following exposure to 30 J/m^2 of local UV irradiation through an 8 µm pore size filter. (D) Removal of 6-4PP in (▲) normal human and (□) XP-E cells following exposure to 30 J/m^2 of global UV. The dotted line is taken from C showing removal of 6-4PP in XP-E cells which were locally UV-irradiated with 30 J/m^2 and is depicted as a reference. Signal intensities were determined by measuring the total fluorescence intensity of a spot or a nucleus, and dividing by the surface area of the measured spot or nucleus, respectively.



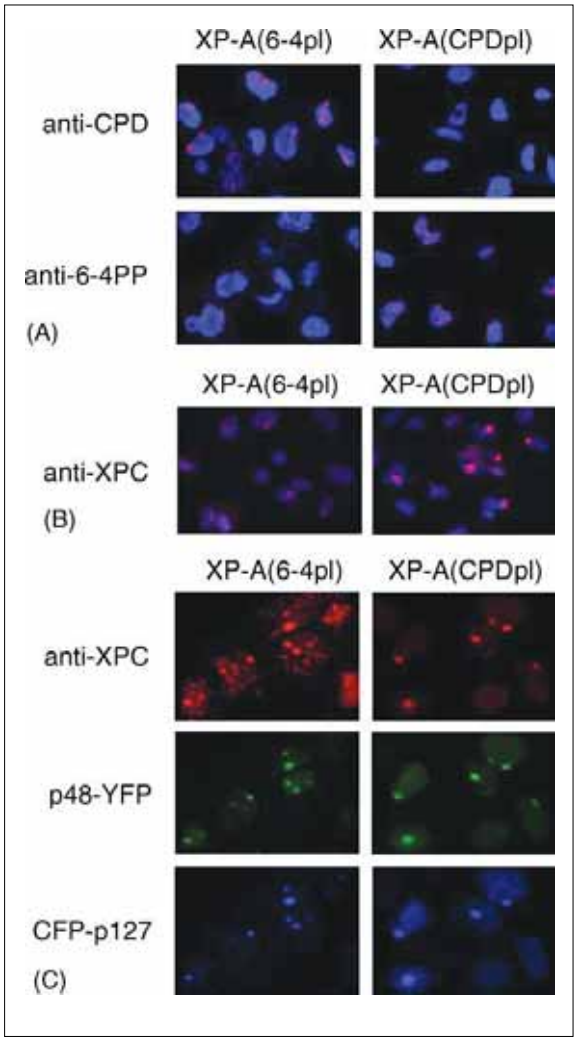
Chapter 9, Figure 5. XPC accumulates differentially in normal human fibroblasts (VH10hTert) and XP-E fibroblasts (GM01389hTert) after local UV irradiation

Cells were UV-irradiated with 20 J/m^2 through an 8 µm pore size filter. (A) Fluorescent immunostaining of XPC (red) at various times after irradiation. Arrows indicate protein accumulations in UV-damaged spots that are poorly visible. Dotted lines outline the nuclei as determined from DAPI nuclear counterstaining (images not shown). All fluorescent images were taken with equal exposure times. (B) Bar graph showing average intensities of XPC spots at various time points after irradiation (blue bars: normal cells; red bars: XP-E cells). Spot intensities were determined by measuring the total fluorescence intensity of a spot, divided by its surface area, and corrected for background levels of fluorescence in the nucleus. For each time point, at least 20 cells were measured. The fluorescent signal measured 30 min after UV in normal human cells was set to 100%.



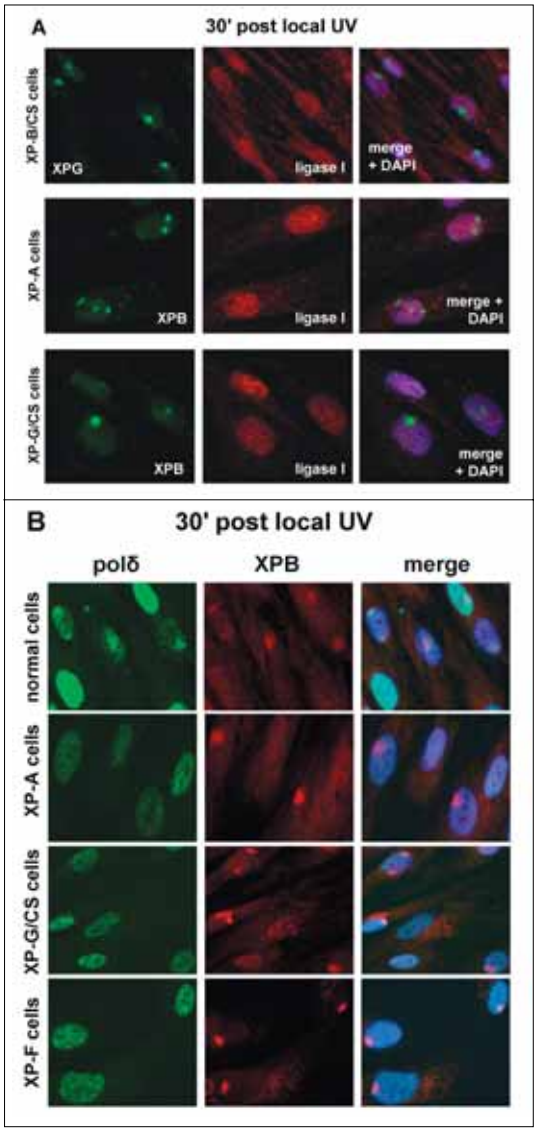
Chapter 9, Figure 7. Formation of p48 spots after induction of local UV damage coincides with 6-4PP repair

Normal human cells (MRC5) stably expressing p48-YFP were exposed to 30 J/m^2 of UV through a 5 µm pore size filter. (A) Fluorescent images of p48-YFP (green) merged with DAPI nuclear DNA staining (blue) taken at various times following irradiation. Arrows indicate sites of local UV damages. Pictures were taken with equal exposure times. (B) Fluorescent immunostaining using anti-CPD antibody (red) or anti-6-4PP antibody (red) 4 h after exposure, merged with DAPI nuclear DNA staining (blue).



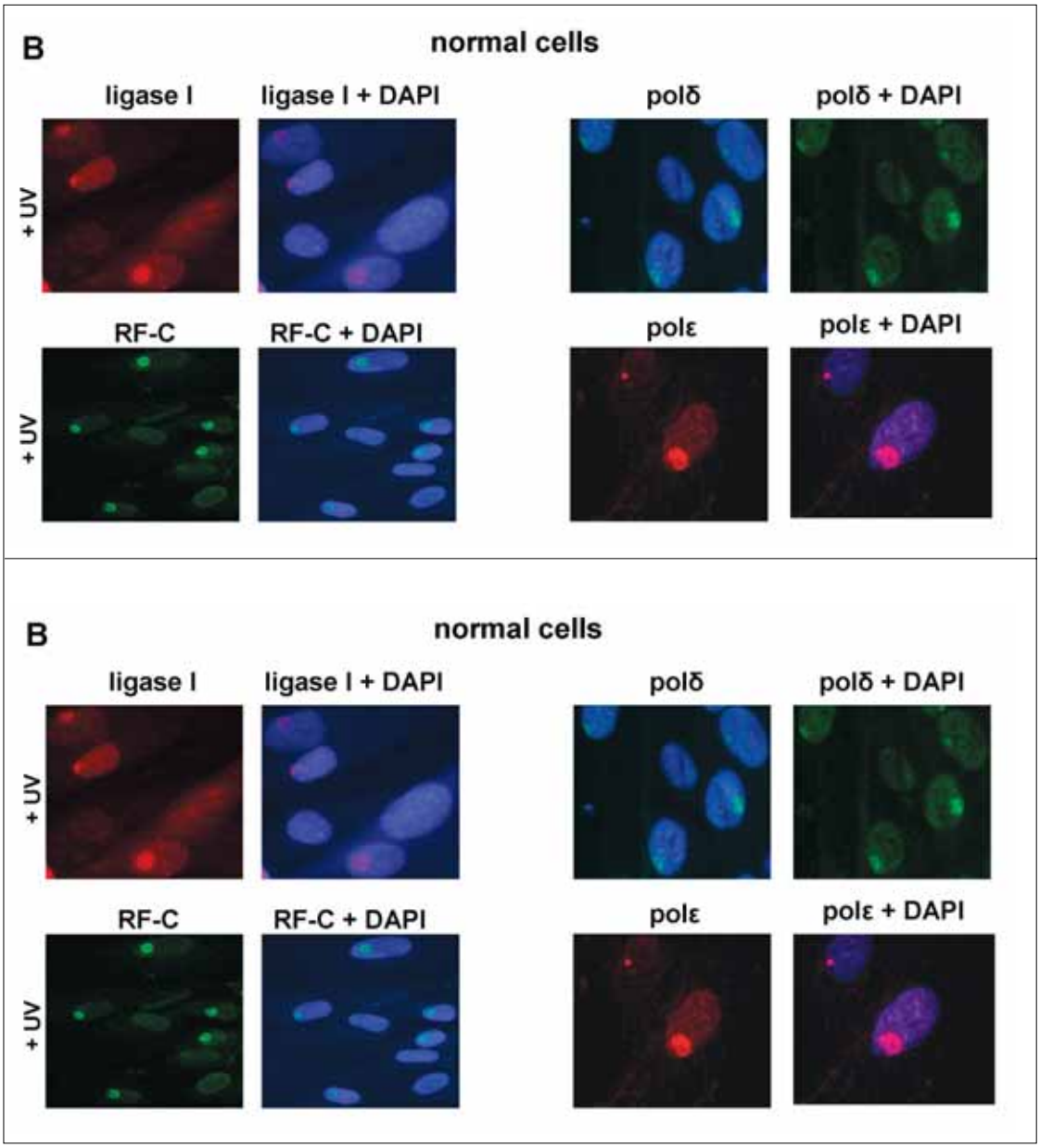
Chapter 9, Figure 8. Accumulation of XPC, p48 and p127 in local UV-damaged spots after removal of one of the two types of UV-photolesion by photoreactivation

XP-A cells stably expressing 6-4PP photolyase [XP-A(6-4)phl)] or CPD photolyase [XP-A(CPDphl)], respectively, were irradiated with 30 J/m^2 of UV through a 5 µm pore size filter, and exposed to photoreactivating light for 1 h. (A) Fluorescent immunostaining of CPD (red) and 6-4PP (red), merged with DAPI nuclear DNA staining. (B) Fluorescent immunostaining of XPC after photoreactivation of 6-4PP (left) or CPD (right) in XP-A photolyase expressing cells. Pictures taken with equal exposure times and merged with DAPI nuclear counterstaining (blue). (C) XP-A(6-4)phl or XP-A(CPDphl) cells transfected with p48-YFP and CFP-p127 expression constructs 24 h prior to local UV-exposure and 1 h photoreactivation. Fluorescent images of XPC (using anti-XPC antibody, red), p48-YFP (green) and CFP-p127 (blue), taken with exposure times optimised for image clarity.



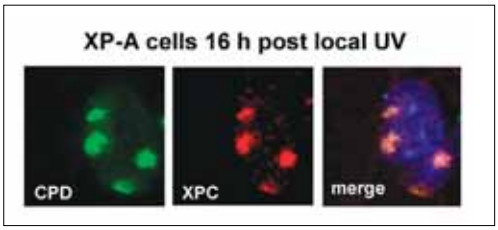
Chapter 10, Figure 2. Differential redistribution of pre- and postincision NER proteins in normal human and XP cells

NER proteins were immunofluorescently labelled in normal human and various repair-deficient XP cells 30 min following local UV treatment with 25 J/m^2.

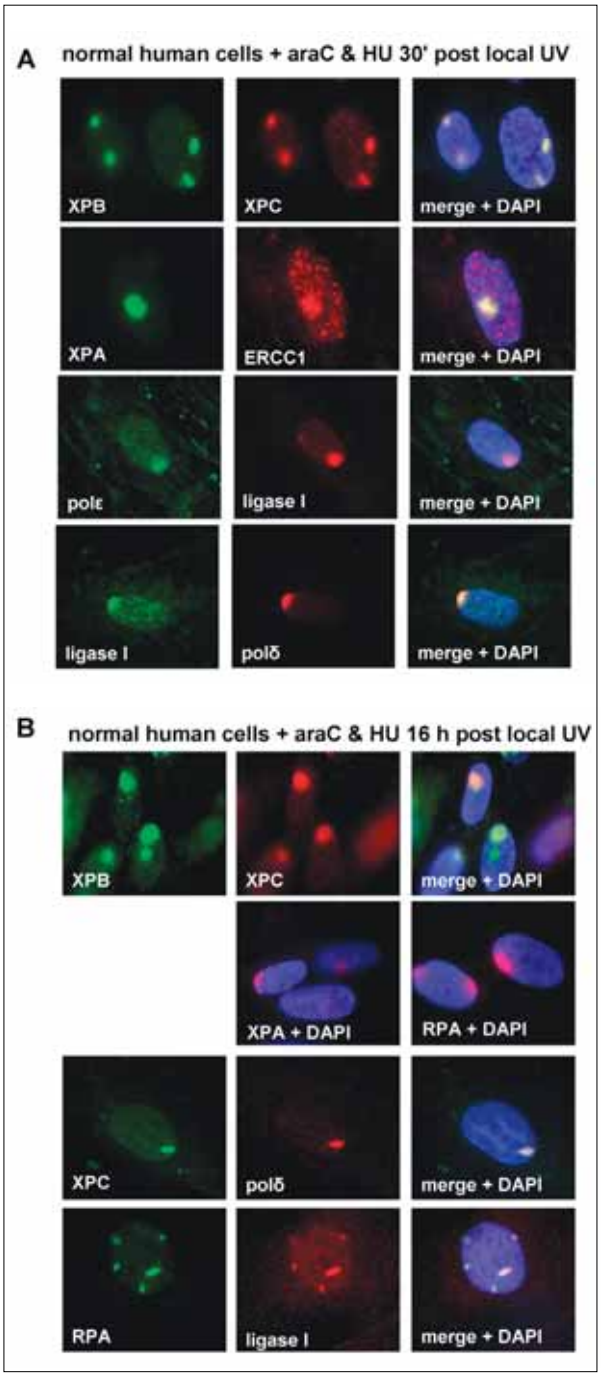


Chapter 10, Figure 1. Involvement of RF-C, polδ, polε and ligase I in NER

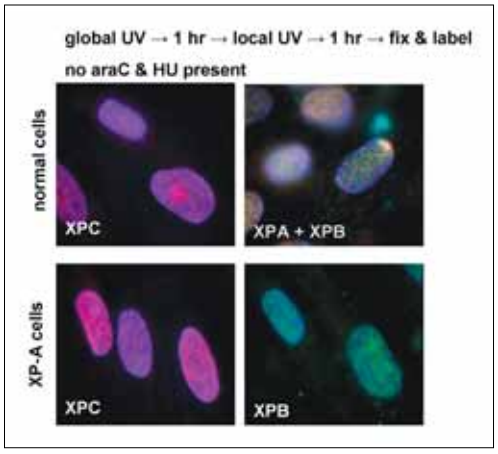
RF-C, polδ, polε and ligase I were immunofluorescently labelled in normal human cells. A, RF-C, polδ, polε and ligase I show a homogeneous distribution pattern prior to UV irradiation. B, 30 min following local UV irradiation with 25 J/m^2 RF-C, polδ, polε and ligase I accumulate at sites of local UV damage.



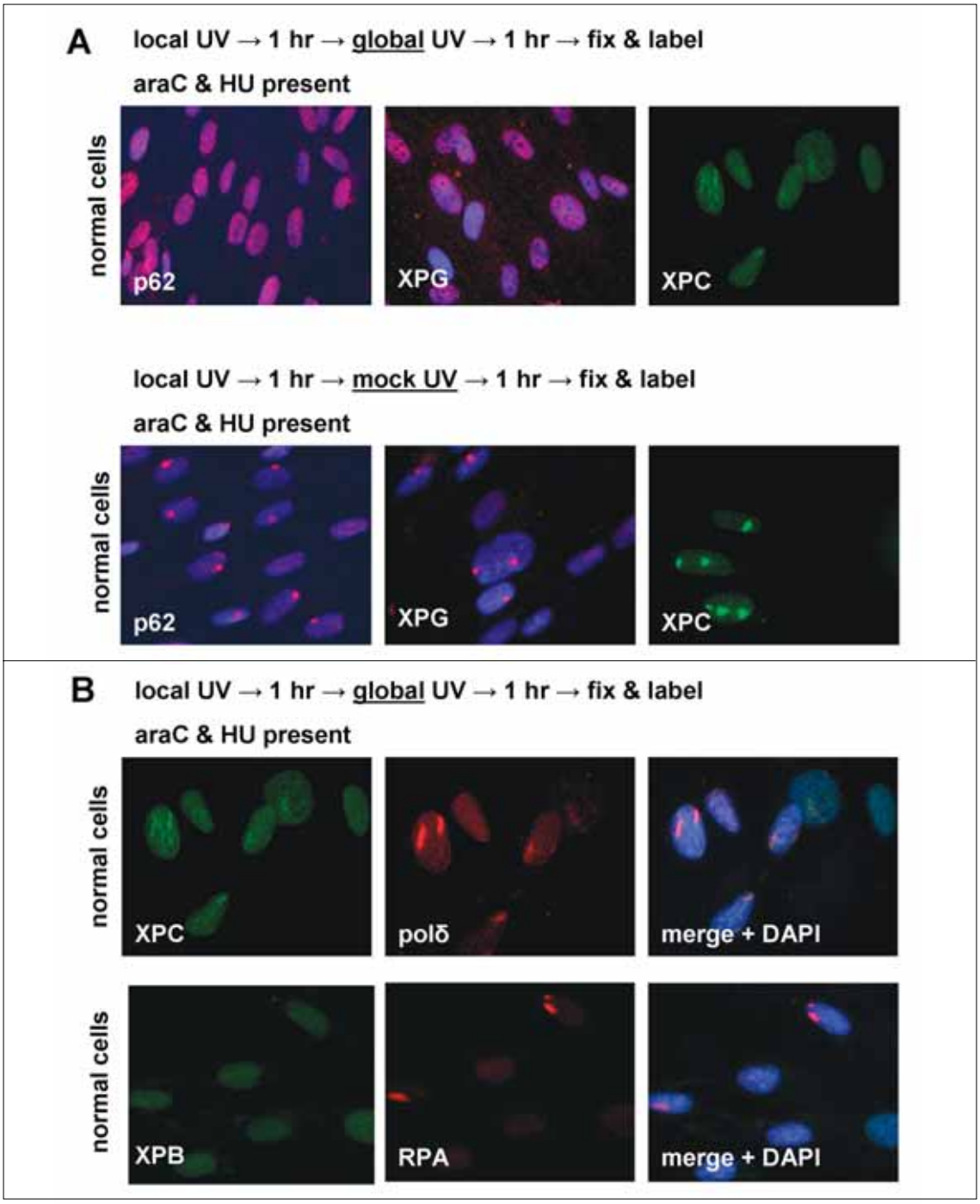
Chapter 10, Figure 3. Sustained accumulation of XPC in NER-deficient cells
XPC and CPD were immunofluorescently labelled in XP-A cells 16 hours after 25 J/m^2 local UV irradiation.



Chapter 10, Figure 4. Rapid and prolonged accumulation of NER proteins in normal human cells in the presence of araC and HU
NER proteins were immunofluorescently labelled following local UV irradiation with 25 J/m^2 in the presence of araC and HU. A, 30 min after UV. B, 16 hours after UV.



Chapter 10, Figure 5. Stability of preincision NER proteins in the NER complex in the absence of araC and HU
Normal human or XP cells were globally irradiated with 25 J/m^2, incubated for 1 hour, locally irradiated with 25 J/m^2 and incubated for another 1 hour before NER proteins were immunofluorescently labelled.



Chapter 10, Figure 6. Stability of preincision and postincision NER proteins at the site of DNA lesions in the presence of araC and HU

Normal human cells were locally irradiated with 25 J/m^2, incubated for 1 hour, globally irradiated with 25 J/m^2 and incubated for another 1 hour before NER proteins were immunofluorescently labelled. A, preincision NER proteins are tethered away from sites of primary UV damage by competition with the secondary UV lesions. B, NER proteins involved in the postincision stages remain stably associated with the primary UV lesions.