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

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Chapter 10

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

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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

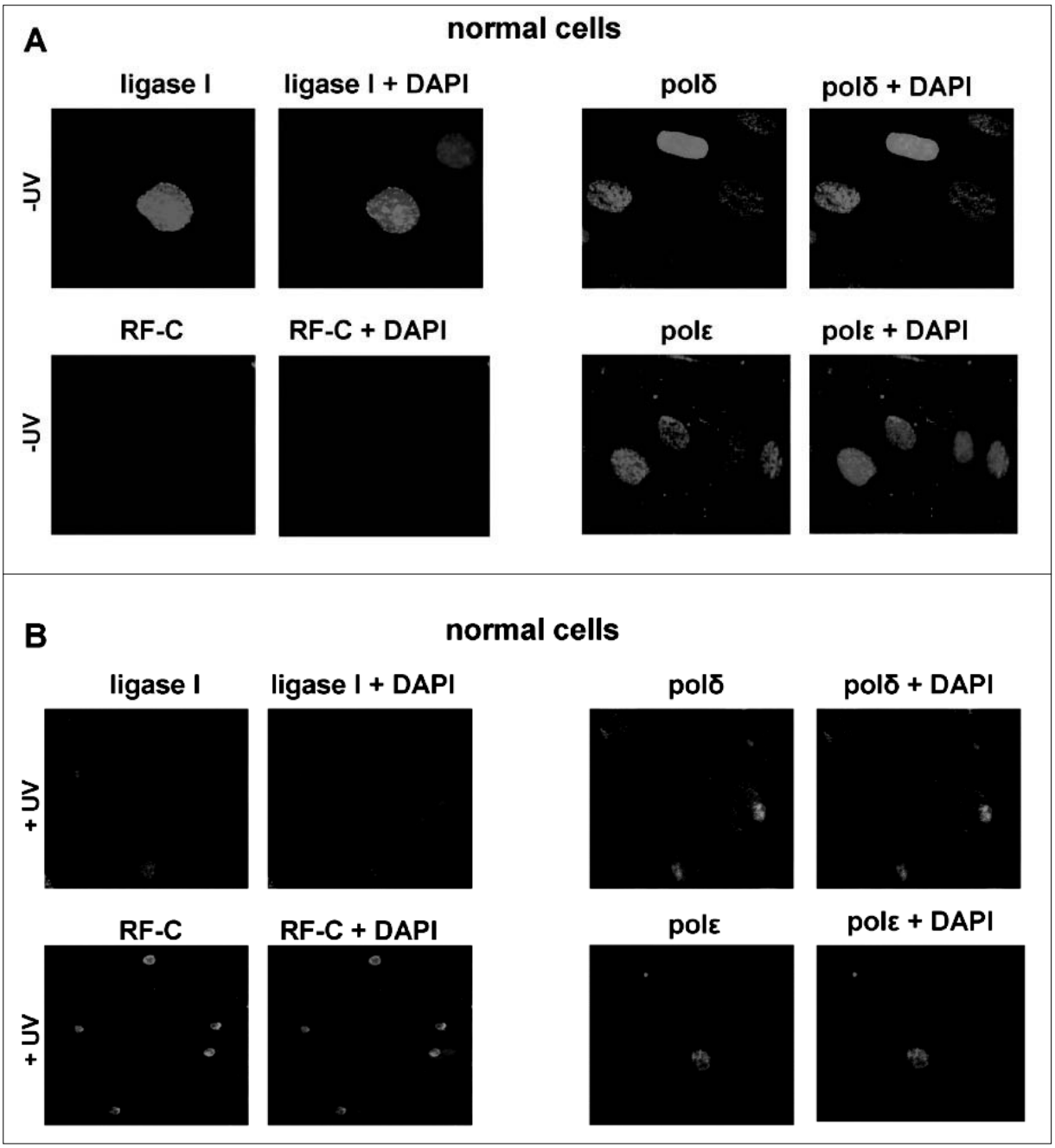

Figure 1. Involvement of RF-C, polδ, polε and ligase I in NER
RF-C, polδ, polε and ligase I were immunofluorescently labelled in normal human cells. A, RF-C, polδ, polε and ligase I show a homogeneous distribution pattern prior to UV irradiation. B, 30 min following local UV irradiation with 25 J/m^2 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

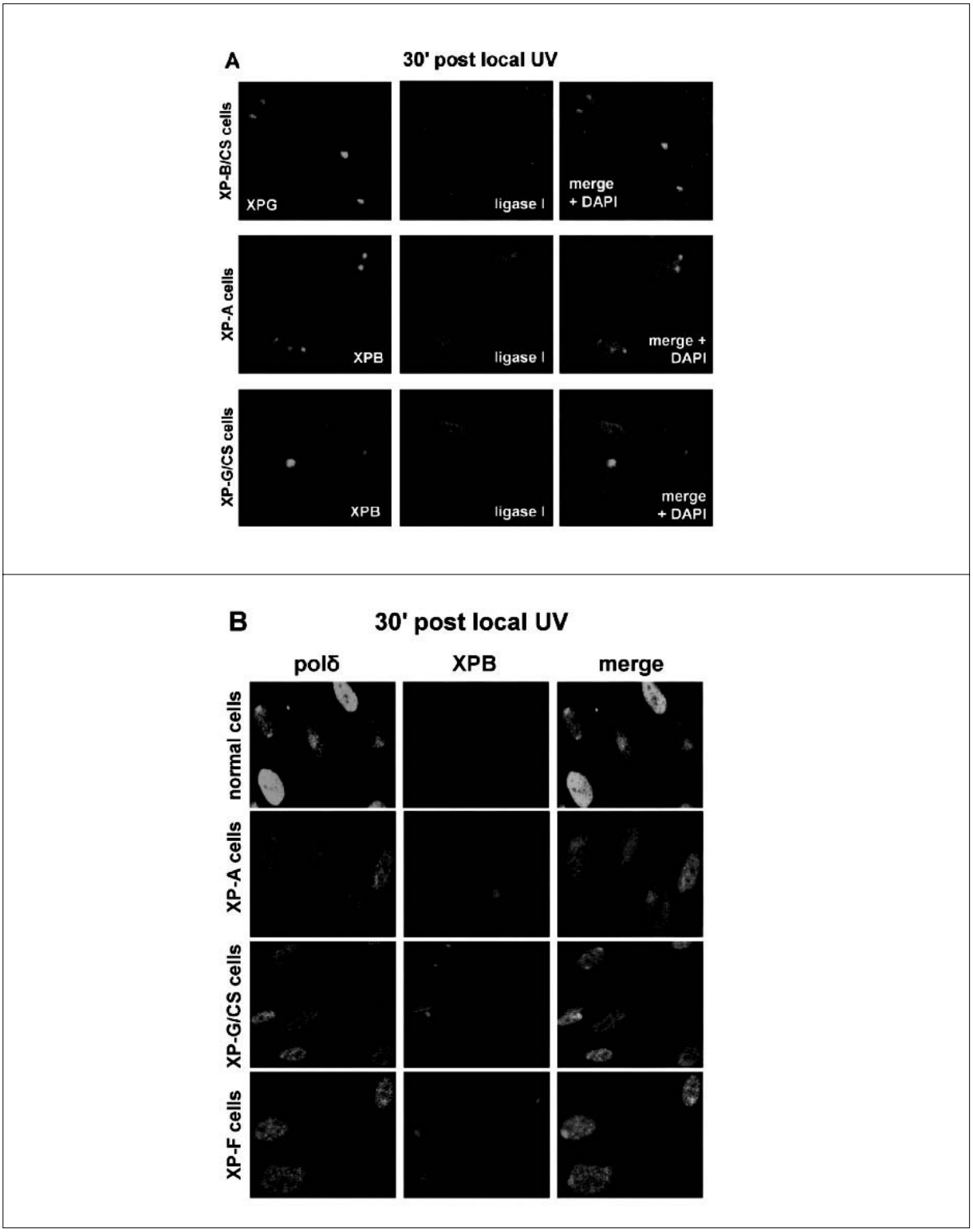

Figure 2. Differential redistribution of pre- and postincision NER proteins in normal human and XP cells
NER proteins were immunofluorescently labelled in normal human and various repair-deficient XP cells 30 min following local UV treatment with 25 J/m^2. See the Appendix for a colour version of this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

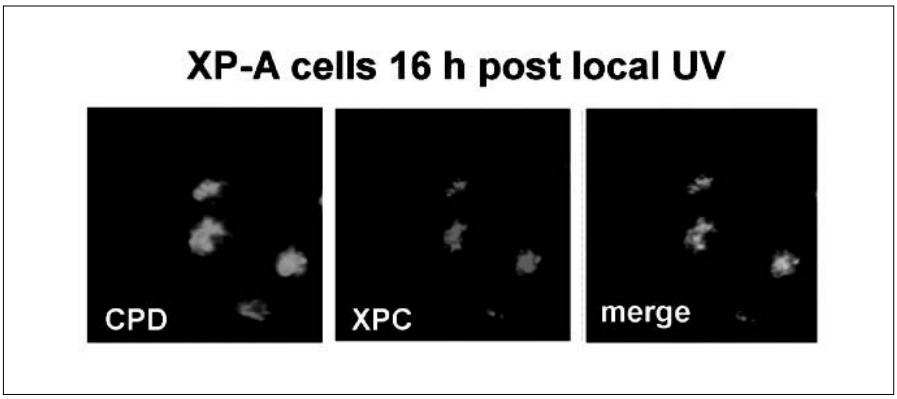

Figure 3. Sustained accumulation of XPC in NER-deficient cells
XPC and CPD were immunofluorescently labelled in XP-A cells 16 hours after 25 J/m^2 local UV irradiation. See the Appendix for a colour version of this figure.

Competition experiments in normal human cells in the presence of inhibitors were carried out by initial local UV irradiation of 25 J/m^2 followed (after a 1-hour incubation period) by either a global UV irradiation of 25 J/m^2 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.

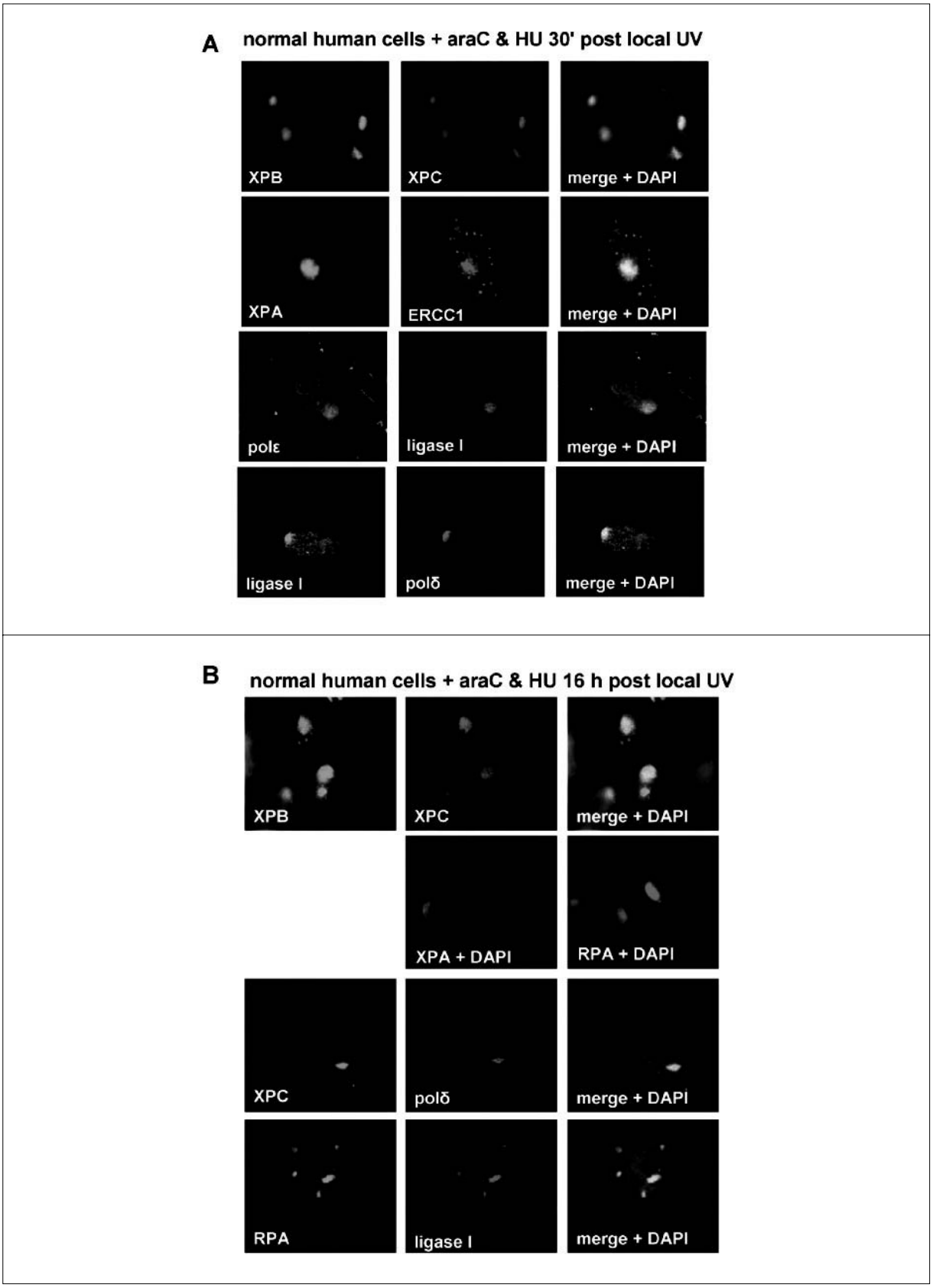

Figure 4. Rapid and prolonged accumulation of NER proteins in normal human cells in the presence of araC and HU
NER proteins were immunofluorescently labelled following local UV irradiation with 25 J/m2 in the presence of araC and HU. A, 30 min after UV. B, 16 hours after UV. See the Appendix for a colour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

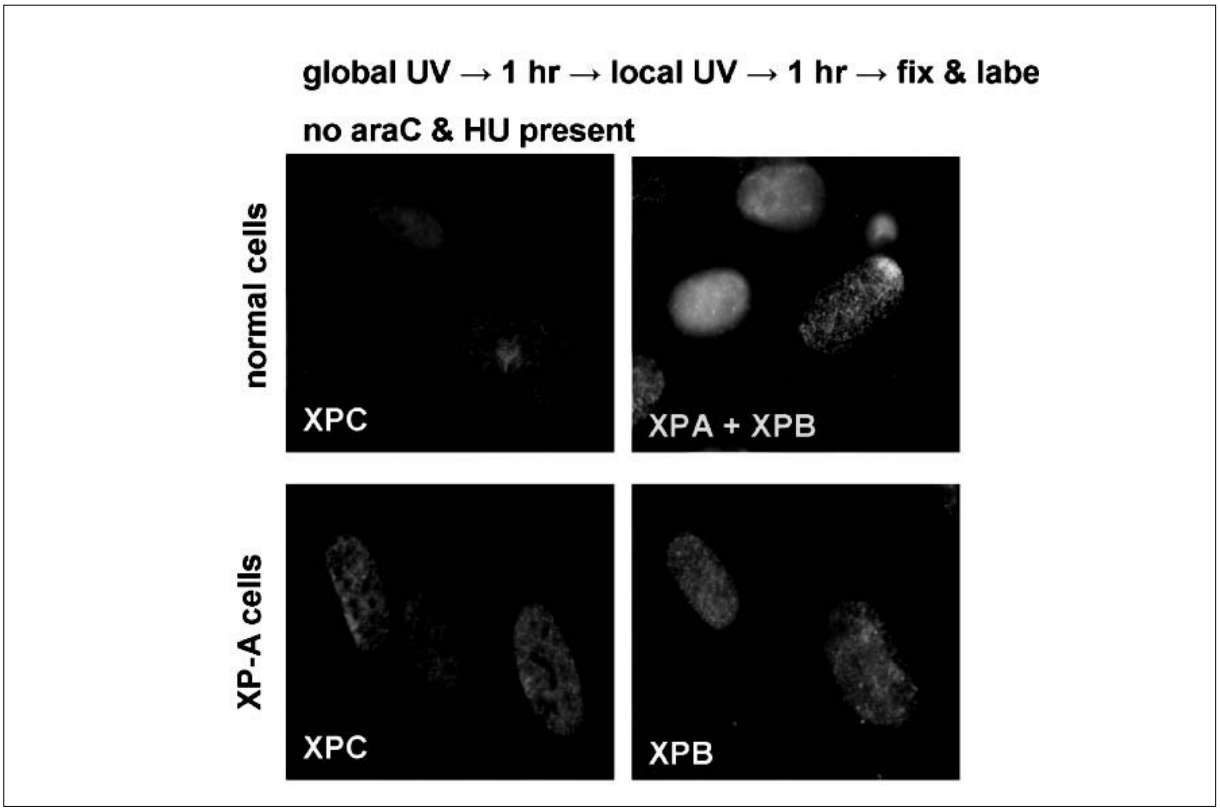

Figure 5. Stability of preincision NER proteins in the NER complex in the absence of araC and HU
Normal human or XP cells were globally irradiated with 25 J/m^2, incubated for 1 hour, locally irradiated with 25 J/m^2 and incubated for another 1 hour before NER proteins were immunofluorescently labelled.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

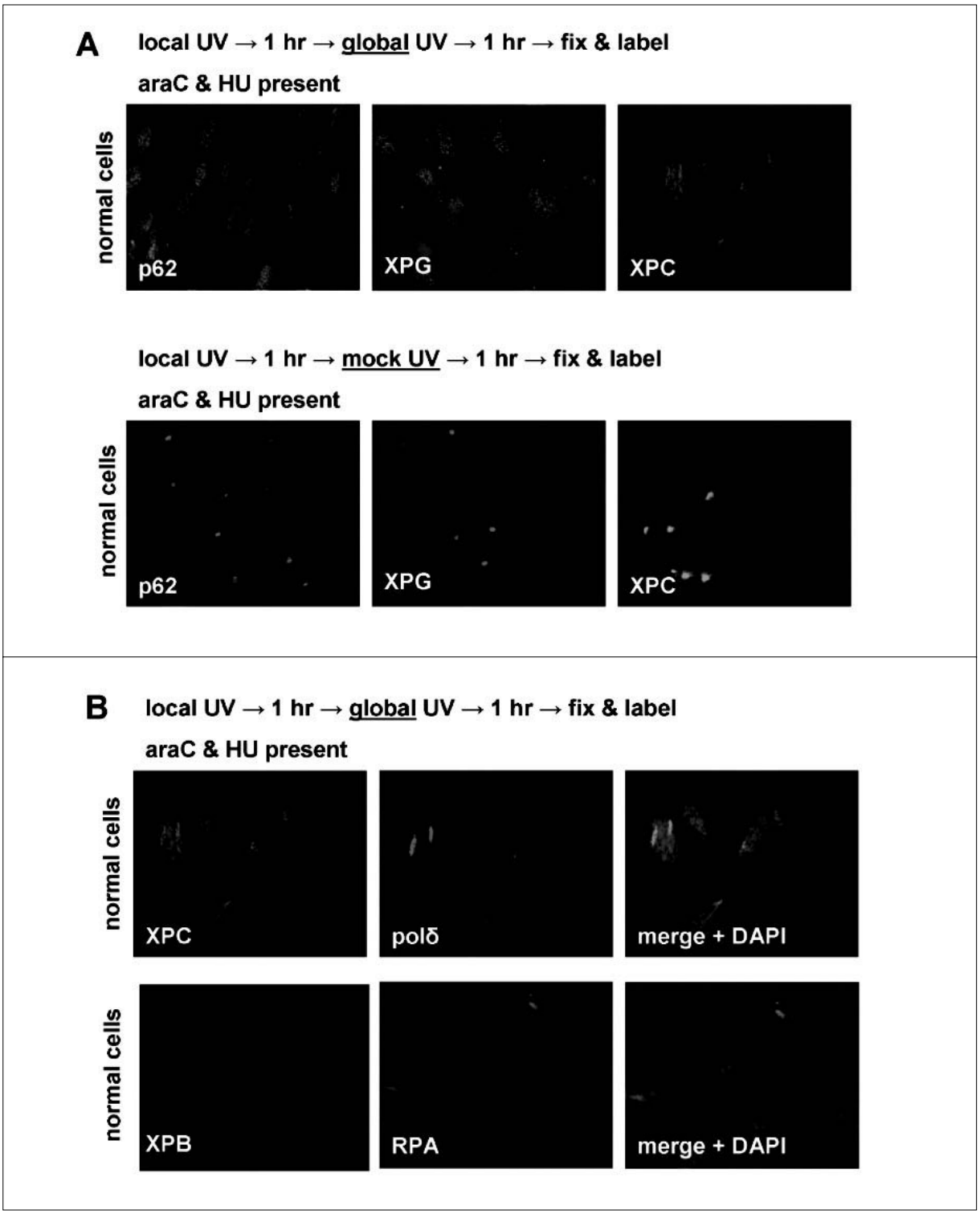

Figure 6. Stability of preincision and postincision NER proteins at the site of DNA lesions in the presence of araC and HU
Normal human cells were locally irradiated with 25 J/m^2, incubated for 1 hour, globally irradiated with 25 J/m^2 and incubated for another 1 hour before NER proteins were immunofluorescently 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. 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 ^{o}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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