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

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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.

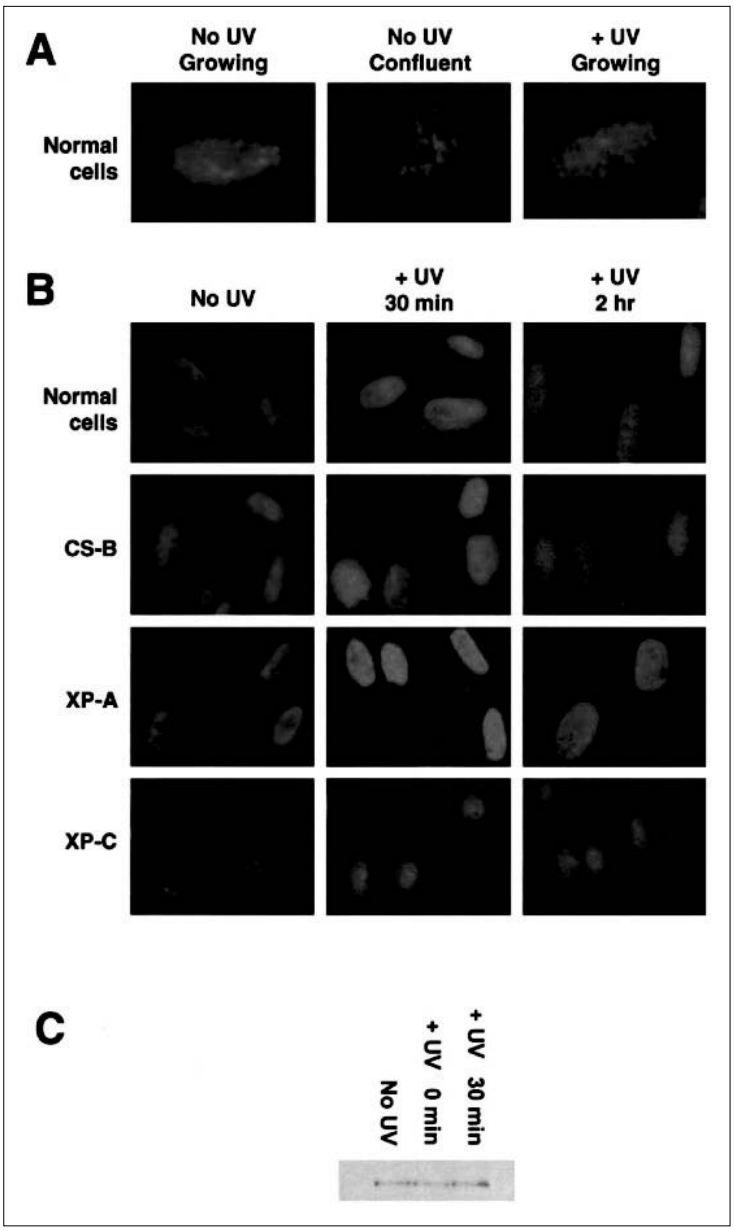
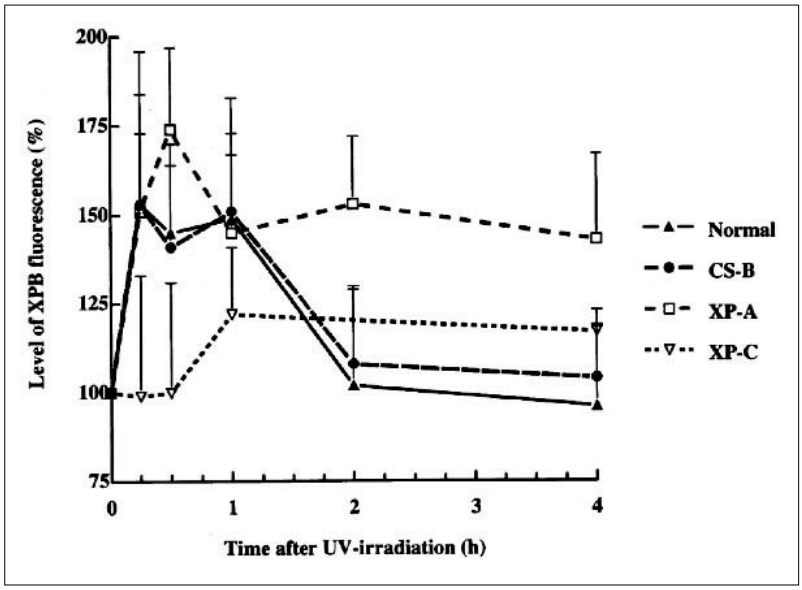


Figure 1. Altered Nuclear Distribution of TFIIH in Confluent Human Fibroblasts after Global UV Irradiation
(A) Growing or confluent normal human fibroblasts (VH25) were fixed and immunolabeled employing an antibody against XPB or immunolabeled at 30 min after UV irradiation (10 J/m^2). (B) Confluent 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

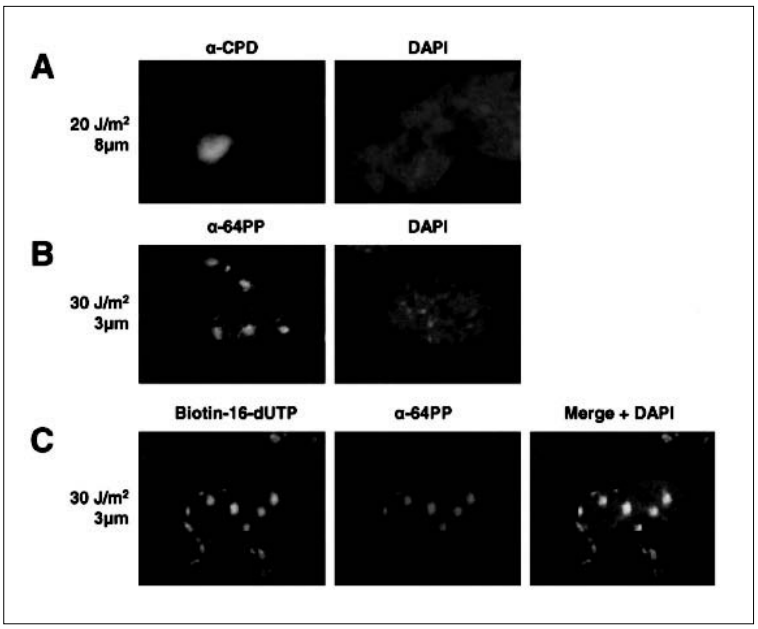


Figure 3. UV Exposure through Isopore Polycarbonate Filters Causes Locally Damaged Areas in the Nuclei
Normal human fibroblasts (VH25) were UV-irradiated with 20 or 30 J/m^2 through a 3 or 8 μm pore filter and immediately fixed. Immunofluorescent labeling was performed using (A) an antibody against CPD (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.

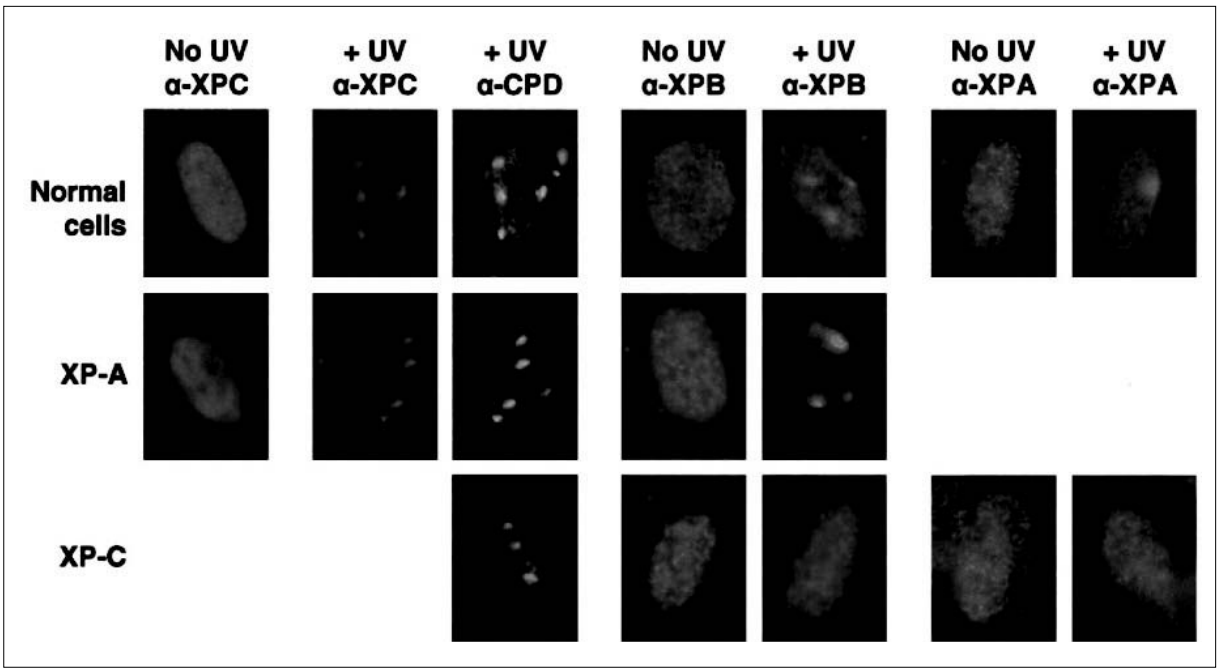


Figure 4. Recruitment of NER Proteins to Sites of UV Damage
Normal human (VH25), XP-A (XP25RO), and XP-C (XP21RO) fibroblasts were UV exposed to 30 J/m^2 through 3 µm filters or mock irradiated and at 15 min after exposure immunolabeled with an antibody (red) against XPC and an antibody (green) against 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,

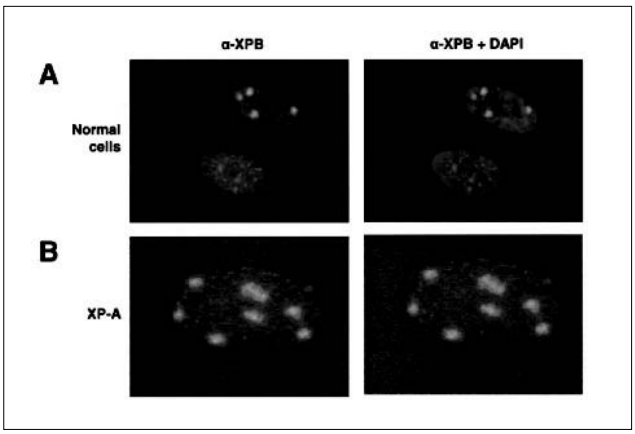


Figure 5. Reduction of XPB in Unexposed Parts of the Nucleus after Local UV Irradiation
Normal human fibroblasts (VH25) (A) or XP-A cells (XP25RO) (B) were UV irradiated with 30 J/m^2 through 3 μm pore filters and immunolabeled for XPB 15 min or 4 hr after UV exposure, respectively. See the Appendix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

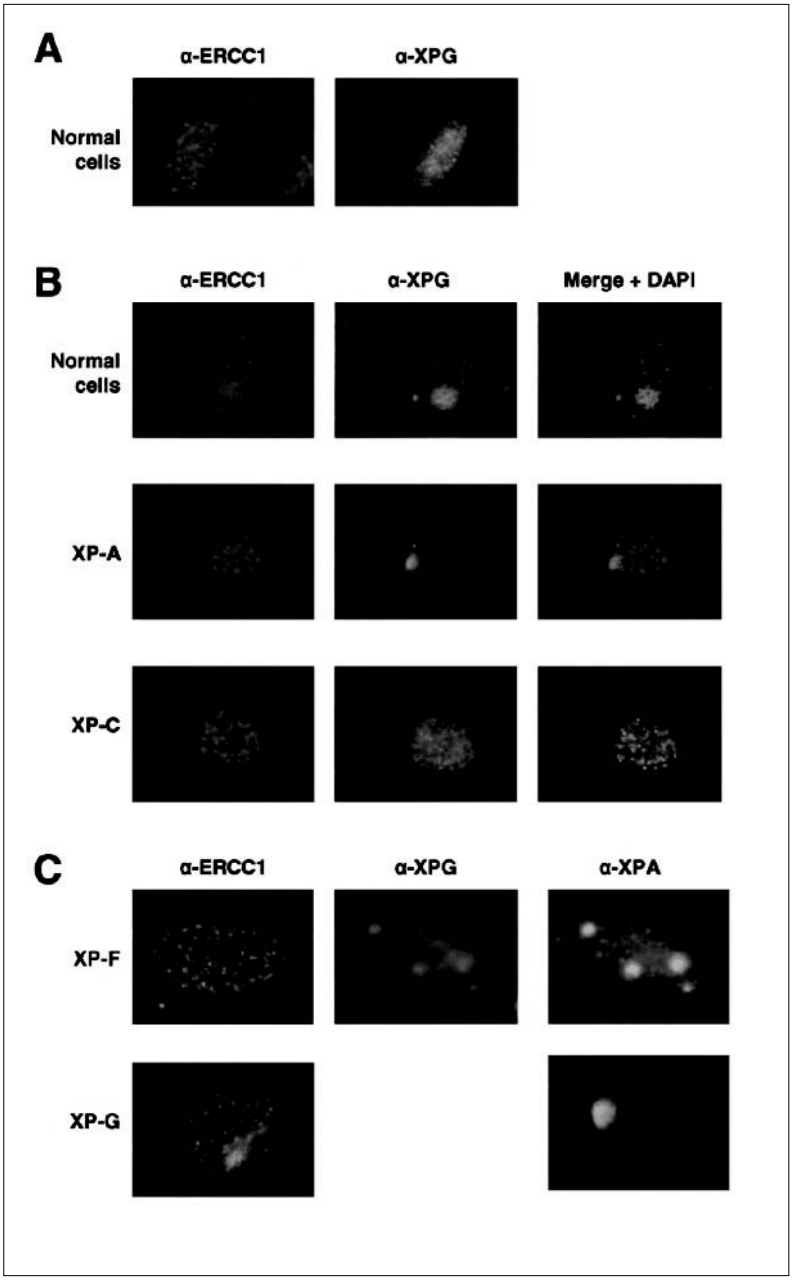


Figure 6. Recruitment of the NER Endonucleases to the Sites of UV Damage
Confluent fibroblasts were UV irradiated (30 J/m^2) through 3 µm filters or mock irradiated, fixed 15 min after UV exposure and immunolabeled for ERCC1, XPG, or XPA. (A) Unexposed normal human fibroblasts (VH25) were immunolabeled 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).

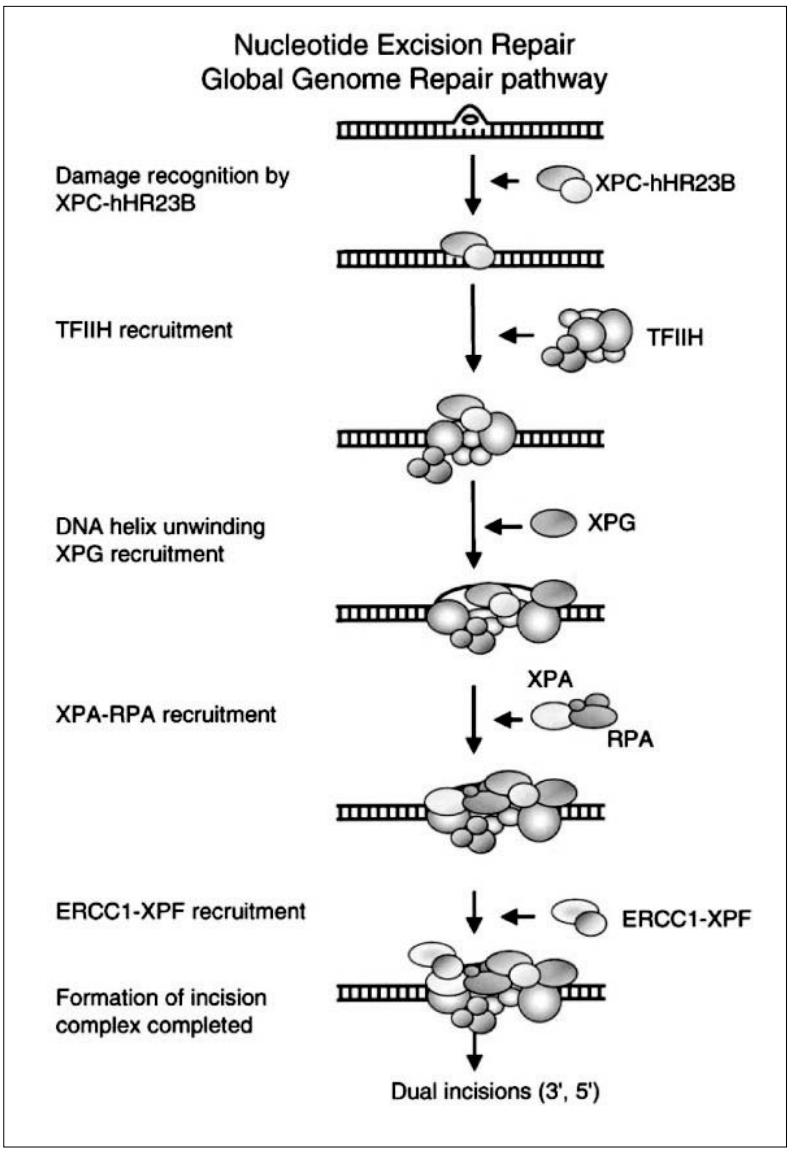


Figure 7. Model for the Assembly of the Human NER Incision Complex

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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