The Spacer Region of XPG Mediates Recruitment to Nucleotide Excision Repair Complexes and Determines Substrate Specificity*

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XPG has structural and catalytic roles in nucleotide excision repair (NER) and belongs to the FEN-1 family of structure-specific nucleases. XPG contains a stretch of over 600 amino acids termed the “spacer region” between the conserved N- and I-nuclease regions. Its role is unknown, and it is not similar to any known protein. To investigate its possible functions, we generated and analyzed several deletion mutants of XPG. The spacer region is not required for endonuclease activity, but amino acids 111–550 contribute to the substrate specificity of XPG, and they are required for interaction with TFIIH and for NER activity in vitro and in vivo. Deletion of residues 184–210 and 554–730 leads only to a partial defect in NER activity and a weakened interaction with TFIIH. XPGΔ184–210 and XPGΔ554–730 are not observed at sites of local UV damage in living cells by immunofluorescence, suggesting that the weakened interaction between XPG and TFIIH results in an NER reaction with altered kinetics. This study demonstrates that the N-terminal portion of the spacer region is particularly important for NER progression by mediating the XPG-TFIIH interaction and XPG substrate specificity.

Nucleotide excision repair (NER) is a remarkably flexible DNA repair pathway with the ability to eliminate a plethora of diverse DNA lesions caused by numerous environmental agents (1, 2). The hallmark of good NER substrates is that they induce significant helical distortion in addition to a chemical change in the DNA structure (3, 4). Versatility as well as specificity in NER are achieved through sequential and highly coordinated action of the various factors involved. The importance of NER is illustrated by the dramatic consequences of a defect in many of the approximately 30 genes involved, resulting in the three disorders xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (5, 6). Among these, XP represents a classical repair syndrome that is characterized by extreme UV sensitivity and a high incidence of skin cancer associated with sunlight exposure.

After initial damage recognition in NER, the helix is opened around the lesion, the damaged strand is incised on both sides of the lesion and released as a 24–32-nucleotide-long oligonucleotide, and the resulting gap is then filled in by repair synthesis (7). A subpathway of NER preferentially removes damage from the transcribed strand of active genes. This transcription-coupled repair process remains poorly understood but is thought to be initiated by a stalled transcribing RNA polymerase at the lesion (8). In contrast, the initial damage recognition and incision steps of NER in bulk DNA, sometimes referred to as global genome repair, are understood in some detail. This process has been reconstructed in vitro using the six repair factors XPC-HR23B, XPA, RPA, TFIIH, XPG, and ERCC1-XPF (9–11). XPC-HR23B appears to be the initial sensor of helix-distorting lesions and is essential for the sequential assembly of all subsequent NER factors (12, 13). XPC-HR23B directly recruits the transcription and repair factor TFIIH, which initiates opening of the DNA around the lesion by the action of its two helicase subunits XPB and XPD (14, 15). Subsequently, XPA, RPA, and XPG join the complex, leading to the formation of a fully opened and stable pre-incision complex (16–18). XPA, RPA, and XPG are recruited independently of each other to sites of UV damage, and addition of ERCC1-XPF to the complex requires XPA but not XPG (13, 19). However, the presence of XPG without XPA and RPA is apparently insufficient to allow the 3’ incision. Likewise, the physical presence of XPG is required for the 5’ incision activity of ERCC1-XPF (20, 21), indicating that although the two endonucleases are recruited to pre-incision complexes independently of each other, the proper assembly of all the factors is required for dual incision around the lesion and progression through the NER pathway.

We have had a long standing interest in elucidating the roles of the XPG protein in NER and other processes (22). The nuclelease function of XPG is required for both NER and transcription-coupled NER, and XPG has an additional nuclelease-independent function in the assembly of NER pre-incision complexes. Structural roles of XPG are also important for additional functions outside of the NER, most likely transcription (23) and the repair of oxidative damage (24, 25). XPG belongs to the FEN-1 family of structure-specific nucleases, whose members share two highly conserved nuclease motifs called N- and I-regions (26). In FEN-1 and most other family members, the N- and I-regions are separated by only

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The abbreviations used are: NER, nucleotide excision repair; AAF, aminoazetylfluorene; ss, single strand; ds, double strand; BSA, bovine serum albumin; PBS, phosphate-buffered saline; XP, xeroderma pigmentosum; CS, Cockayne syndrome; GFP, green fluorescent protein; 6-4PPs, (6-4) photoproducts; RPA, replication protein A.

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about 70 amino acids, which form a flexible helical loop in the proteins with known structures (27–30). In contrast, the N- and I-regions in XPG are separated by a stretch of over 600 amino acids that was designated “spacer region” (31). This region does not contain any known structural motifs, but it has been speculated that it has a role in mediating interactions with proteins involved in NER and other processes. TFIIH appears to be the key interacting partner for XPG in NER, and it needs to be present at damage sites for the subsequent recruitment of XPG (13, 32). The interaction between XPG and TFIIH is sufficiently strong that the two proteins co-purify from cell extracts over several chromatographic steps (10). They can also be co-immunoprecipitated and then used in NER reactions missing these two components (15). Pull-down assays using in vitro translated proteins have shown that XPG fragments, including parts of the spacer region, interact with the XBP, XPD, p62, and p44 subunits of TFIIH (33). The interacting domains have not yet been precisely localized, but 7 amino acids near the N terminus of the spacer region are known to be important for the TFIIH interaction (34). Additionally, an RPA-XPG interaction has been mapped to the spacer region by using in vitro translated proteins, but the functional significance of this reported interaction is not yet clear (35).

In addition to mediating protein-protein interactions, the spacer region may also contribute to the substrate specificity of XPG. FEN-1 and XPG show distinct but overlapping substrate specificities in vitro, cleaving various DNA substrates with single-strand (ss)/double-strand (ds) DNA junctions adjacent to the 5'-ssDNA overhang. Whereas FEN-1 and other members of this nuclease family need a single-stranded 5' end for activity (36–38), XPG efficiently processes bubble substrates, which have no free 5' ends (39, 40). Given the involvement of XPG and FEN-1 in different repair and replication pathways, it is possible that the XPG-specific spacer region may be a determinant for the unique substrate specificity of XPG. These observations suggest that the spacer region of XPG has an important role in NER by mediating association with other NER factors and by contributing to the substrate specificity of XPG. Here we tested these hypotheses by generating mutants of XPG with deletions in the spacer region and by analyzing their properties at the biochemical and cell biological levels. Our studies reveal that the spacer region of XPG is dispensable for endonuclease activity but is required for efficient bubble cleavage activity, interaction with TFIIH and NER activity. We discuss these results in the context of how XPG contributes to the progression through the NER incision reaction as a modular protein.

**EXPERIMENTAL PROCEDURES**

**Construction of XPG Spacer Deletion Mutants**—Deletion mutants were generated by digestion of pFastBac1-XPG (41) with HindIII and NdeI (Δ111–550), with NdeI and Asel (Δ565–730), or with HindIII and Asel (Δ111–730), and the resulting pFastBac1-XPG fragments were ligated with a double-stranded adaptor DNA having corresponding sticky ends. The adaptors were made by annealing two oligonucleotides, and the resulting pFastBac1-XPG fragments were ligated with a double-stranded adaptor DNA having corresponding sticky ends. The adaptors were made by annealing two oligonucleotides having the sequence 5'-AAGAAAGGACTTAGCG and (B) 5'-GAAAGGATTATGAAAGAACTCTTCC. Two separate PCRs were performed. This latter PCR results in the deletion of the spacer region are known to be important for the TFIIH interaction (34). Additionally, an RPA-XPG interaction has been mapped to the spacer region by using in vitro translated proteins, but the functional significance of this reported interaction is not yet clear (35).

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Functional Characterization of the XPG Spacer Region

50 μM DCTP) and incubated for another 12 min at 37 °C. Reactions were stopped by addition of 8 μl of formamide loading dye, heated at 95 °C for 5 min, and placed on ice. Reactions were loaded on a 14% sequencing gel and run in 1× TBE at 60 watts. The MapI-digested pBR322-XPG clones were used as size markers after end-labeling with [α-32P]dCTP and Klenow enzyme (New England Biolabs). The gels were exposed on phosphor screens and scanned on the PhosphorImager.

Cell Transduction with Lentiviral Recombinants—Recombinant lentiviruses containing the various XPG cDNAs under the control of the EF1α promoter were produced by co-transfecting 293T cells with the following three plasmids: the packaging plasmid R591, the vesicular stomatitis virus-G envelope-protein expression plasmid pMDG, and the pLX/O/EW lentiviral vector containing the different XPG cDNAs (49). A plasmid containing the green fluorescent protein (GFP) cDNA was used as negative control. Details of the lentiviral vectors and protocols are described on the following website: www.tronolab.unige.ch. Briefly, the culture medium containing the lentiviral XPG recombinants was harvested and filtered through a 0.45-μm polycarbonate membrane filter (Millipore). Infection was carried out by adding the viral particles containing the different recombinants to XP/GCS primary fibroblasts (XP20BE) at 50% confluency. Fibroblasts were then cultured normally for various times, and transduction efficiency was assessed by XPG immunofluorescence.

UV Irradiation and Survival Assay—Cells were seeded on dishes and cultured overnight prior to UV irradiation. Irradiation was performed using a TUV 6-watt lamp (UVGL-58, Omnilab), and the UVC dose rate was measured by using a UV radiometer (UVX-25, Omnilab). 50–60% confluent fibroblasts were irradiated with increasing doses of UVC, and cell viability was assessed 72 h post-irradiation using the CellTiter 96 AQUANT One Solution Cell Proliferation Assay (Promega) according to the manufacturer’s instructions. The number of proliferating cells was measured by recording the absorbance at 490 nm with a 96-well plate reader (Bio-Rad). Viability assays were performed independently four times using two independent sets of transduced cells.

Local UV Irradiation and Immunofluorescence—Fibroblasts were seeded on glass coverslips and cultured overnight prior to UV irradiation. Cells were either non-irradiated or irradiated with 150 J/m² through 8-μm polycarbonate isopore membrane filters (Millipore). Cells on four coverslips were irradiated under the same filter, and two coverslips were incubated in their original medium and fixed 30 min post-UV. Cells were fixed and permeabilized with 3% formaldehyde, 0.3% Triton X-100 in PBS for 20 min at room temperature. The stabilized XPG was performed in 1× PBS containing 1% BSA for 1 h at room temperature. The primary antibodies, mouse monoclonal anti-XPG (clone SH7) at 1/2000 dilution and rabbit polyclonal anti-rat p88 at 1/600, were added for 1 h at room temperature in 1× PBS containing 1% BSA. Detection of (6-4) photoproducts (6-4PPs) was performed by using the mouse monoclonal antibody 64M-2 against 6-4PPs (50) as described (13), except that cells were treated with 0.7 M HCl instead of 2 M HCl. Detection was performed using the anti-mouse IgG (H + L) AlexaTM 488 conjugate (Molecular Probes) and the anti-rabbit IgG (H + L) Cy3TM conjugate (Jackson Immunoresearch) for 1 h at room temperature, both at 1/600 dilution.

Nuclear staining was performed during the incubation of the secondary antibody using 4,6-diamidino-2-phenylindole staining (Molecular Probes) at 1/300 dilution. Stained cells were observed in a Zeiss AxioVision microscope using the Axiovision software. Transduced cells were analyzed for the presence or absence of UV-induced foci, and untransduced cells were used as negative controls.

Immunoprecipitation and Immunoblotting—XP20BE cells were transduced with lentivectors containing cDNAs encoding wild-type XPG or XPG spacer mutants. Immunoprecipitations were performed as described previously (34) with the following modifications. Cells were lysed for 1 h on ice in lysis buffer (25 mM HEPEs, KOH, pH 7.9, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol) containing 1× complete protease inhibitors (Roche Applied Science). Dynabeads PANT anti-human IgGs (Dynal) were pre-coupled to the SH7 anti-XPG monoclonal antibody at a ratio of about 0.5 μg of antibody per 10⁷ beads for 1 h at room temperature. Cell extracts were incubated with pre-coupled XPG-beads for 2 h at room temperature. Beads were collected using a magnetic particle concentrator (Dynal MPC) and were washed four times in lysis buffer containing 1× complete protease inhibitors (Roche Applied Science). Beads were resuspended in 2× loading buffer and analyzed by SDS-PAGE and immunoblotting. Samples were fractionated by SDS-PAGE and immunoblotted as described previously.

RESULTS

Construction and Purification of XPG Spacer Deletion Mutants—We constructed several deletion mutants in the spacer region—between the N- and I-domains of XPG using appropriate available restriction enzyme sites, resulting in deletion of either the N-terminal (Δ111–550), C-terminal (Δ554–730), or both parts (Δ111–730) of the spacer. Residues within XPG region 1–337 have been shown to interact with TFIIH (33). This region contains the conserved nuclease N-domain and the N-terminal part of the spacer (Fig. 1A). Residues within the region 668–747 located in the C-terminal part of the spacer have been reported to contain an RPA interaction domain (Fig. 1A) (35). Additionally, we made a deletion of a short sequence termed the D1 box, a stretch of 27 amino acids in the N-terminal part of the spacer region that is conserved in higher eukaryotes (51). Wild-type and deletion mutants of XPG were expressed in S9 insect cells as C-terminal histidine-tagged

FIG. 1. Construction and purification of XPG spacer deletion mutants. A, schematic representation of wild-type XPG, spacer deletion mutants, and FEN-1 primary protein structure. The two nuclease motifs (N- and I-regions) and the interaction sites of XPG with TFIIH and RPA are indicated. The restriction sites that were used to generate the Δ554–730, Δ111–550, and Δ111–730 mutants are indicated. The Δ184–210 (D1 box) deletion mutant was generated by fusion PCR. The number of amino acids (aa), the molecular weight, and the pI values are given on the right side of the scheme. B, XPG proteins were expressed and purified from S9 insect cells as described under “Experimental Procedures” and analyzed on a 12% SDS-polyacrylamide gel. Lane 1, broad range marker (2 μg of protein/band); molecular weights are indicated. Lanes 2–6, 2 μg of wild-type XPG and XPG with deletions in the spacer region. WT, wild type.

(34). Quantification of at least three independent experiments was performed using the Quantity One software, and a representative gel is shown.
proteins and purified as described under “Experimental Procedures” (Fig. 1B). All deletion mutants showed similar solubility as wild-type XPG and in some cases displayed significantly increased expression levels (data not shown). The spacer region of XPG is very acidic in character (21.4% acidic residues, pI 4.5), and its deletion therefore increased significantly the pI of some of the deletion mutants. The low pI of XPG could be one of the reasons why the protein migrates close to 200 kDa on an SDS-polyacrylamide gel, rather than at its predicted mass of 133 kDa. Like the wild-type protein, deletion mutants with a low pI also migrated above their molecular weight, whereas XPGΔ111–730 with a pI of 9.4 migrated close to the expected molecular weight (see Fig. 1B). The low pI of the spacer region may therefore indeed be responsible for the abnormal migration behavior of XPG.

Deletions in the XPG Spacer Region Influence Substrate Specificity—We first wanted to investigate whether the deletion mutants of XPG contained properly folded nuclease domains and retained nuclease activity on model substrates. In a reaction with a splayed arm substrate with a free 5’ end, all of the mutant proteins showed comparable activity to wild-type XPG in the presence of Mg2+ (data not shown) or Mn2+ (Fig. 2A). These results indicate that the XPG deletion mutant proteins contain properly folded nuclease domains and are able to recognize ss/dsDNA junctions like the wild-type protein.

XPG, but not FEN-1, shows cleavage activity on bubble substrates (36, 37, 40), and we wondered to what extent the spacer region in XPG might contribute to bubble cleavage activity. Whereas bubble cleavage activity of XPGΔ184–210 was equal to wild-type levels, we observed a reduction for XPGΔ554–730 (1.6-fold), XPGΔ111–550 (3.3-fold), and XPGΔ111–730 (3.2-fold) (Fig. 2B). Most interestingly, the activity of XPGΔ111–550 and XPGΔ111–730 reached a plateau at around 30% activity, and a further increase in protein concentration up to 50 nM did not lead to higher activity (data not shown). This reduction in bubble cleavage efficiency was not caused by a reduction in DNA substrate binding, because electrophoretic mobility shift assays showed no significant difference in binding affinity of wild-type XPG, XPGΔ111–550, or XPGΔ111–730 proteins on the bubble substrate using the conditions described in A. C, cleavage efficiency of wild-type XPG and deletion mutants on the splayed arm substrate (top) and bubble substrate (bottom) with increasing protein concentration. Substrate and product bands of two independent experiments were quantified. Standard deviations are indicated by error bars.

**FIG. 2.** Endonuclease activity of wild-type XPG and deletion mutants on splayed arm and bubble substrates. A, 2.5 nM splayed arm substrate was incubated with either wild-type (WT) XPG, XPGΔ184–210, XPGΔ554–730, XPGΔ111–550, or XPGΔ111–730 proteins in the presence of 0.5 mM MnCl2 at 30 °C for 90 min and analyzed on a 15% denaturing polyacrylamide gel. The following protein concentrations were used: 0 nM (lane 1), 0.1 nM (lane 2), 0.25 nM (lane 3), 0.5 nM (lane 4), 1 nM (lane 5), 1.75 nM (lane 6), 2.5 nM (lane 7), 5 nM (lane 8), and 10 nM (lane 9). The position of the 5’ 32P label on the substrate is indicated by the asterisk. B, incision activity of wild-type XPG, XPGΔ184–210, XPGΔ554–730, XPGΔ111–550, or XPGΔ111–730 proteins on the bubble substrate using the conditions described in A. C, cleavage efficiency of wild-type XPG and deletion mutants on the splayed arm substrate (top) and bubble substrate (bottom) with increasing protein concentration. Substrate and product bands of two independent experiments were quantified. Standard deviations are indicated by error bars.
whereas those expressing the XPG variants with deletions in the spacer region were visible at UV foci 30 min post-UV (Fig. 5). However, XPB was still found at these foci in the absence of XPG (Fig. 5, untransduced line). These foci represent sites of local UV damage and are also visible. However, these are found in the nuclei of unirradiated cells (34, 54) and do not correspond to sites of local UV damage.

In contrast to the wild-type XPG, none of the XPG proteins with deletions in the spacer region were visible at UV foci 30 min post-irradiation (Fig. 5). However, XPB was still found at such foci in co-localization with 6-4PPs (Fig. 5, WT line). In addition to these bright foci, small punctate specks of XPG staining are also visible. However, these are found in the nuclei of unirradiated cells (34, 54) and so do not correspond to sites of local UV damage.

At 24 h post-UV, wild-type XPG was no longer found in foci but instead was broadly dispersed throughout the nucleus (Fig. 5). As a measure of XPG-mediated repair activity, we monitored the presence of XPB and 6-4PPs in foci at 24 h post-UV. Previous studies (13) have demonstrated that the disappearance of local UV damage foci coincides with completion of repair synthesis activity as measured by biotin-dUTP incorporation. We therefore took the disappearance of XPB and 6-4PPs foci as a measure of completion of NER. In XP20BE cells and in the absence of functional XPG, XPB and 6-4PPs foci persisted after 24 h, whereas they were completely gone in the same cells transduced with wild-type XPG cDNA (Fig. 5) (34). At 24 h post-UV, XPG immunostaining was found broadly dispersed throughout the nucleus in all XPG spacer deletion mutants.

shown a positive nuclear staining for XPG, thereby indicating very efficient transduction of XP-G/CS cells by the various XPG constructs (data not shown).

Proteins from the XP-G/CS patient XP20BE encode severely truncated XPG proteins that are not detectable by immunofluorescence (Fig. 5, untransduced line). In the presence of the wild-type recombinant XPG protein, XPG was visible in bright nuclear foci within 30 min post-UV (Fig. 5, and (34)). In agreement with previous reports (13, 34), XPG co-localized with TFIIH (XPB) at these foci (white arrows in Fig. 5), and XPB was recruited to these foci in the absence of XPG (Fig. 5, untransduced line). These foci represent sites of local UV damage as shown by the co-localization of XPB and 6-4PPs (Fig. 5, WT line). In addition to these bright foci, small punctate specks of XPG staining are also visible. However, these are found in the nuclei of unirradiated cells (34, 54) and do not correspond to sites of local UV damage.

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ing cells that still displayed XPB foci at 24 h but not at 48 h post-UV (data not shown).

The lack of accumulation of foci at UV-damaged sites of XPG/H9004\(^{184-210}\) and XPG/H9004\(^{554-730}\) was somewhat unexpected, because these proteins retained substantial residual repair activity \textit{in vitro} and \textit{in vivo} (Figs. 3 and 4). The observation that XPB and 6-4PPs foci were present at 30 min but not 24 h post-UV, however, suggests that NER had been completed in these cells despite the fact that no distinct XPG foci could be observed at 30 min post-UV. In addition, and in agreement with the survival data, XPB foci disappeared within 1–2 h after UV irradiation in wild-type XPG and XPG/H9004\(^{554-730}\) transductants (data not shown). By contrast, but also consistent with the \textit{in vitro} NER and UV survival data, XPB and 6-4PPs foci persisted at 24 and 48 h in cells expressing XPG/H9004\(^{111-550}\) and XPG/H9004\(^{111-730}\) (Fig. 5 and data not shown).

These results indicate that an intact spacer region of XPG is required for efficient recruitment to sites of UV damage. It is likely that the \(\Delta 184\)–210 and \(\Delta 554\)–730 spacer mutants assemble less stably and/or with slower kinetics at NER sites so that they cannot readily be detected by direct immunofluorescence.

**XPG Spacer Deletion Mutants Have Reduced Affinity for TFIIH—** The failure to detect a clear signal for the recruitment of the XPG spacer deletion mutants to UV-damaged sites is likely due to an impaired interaction of XPG with one or more NER components. Previous studies (13, 33) have demonstrated that TFIIH is required for the recruitment of XPG to NER complexes and that it can interact with residues within XPG regions 1–377 and 747–928. In addition, the \(\Delta 225\)–231 deletion in the XPG protein found in the patient XPCS1BD results in impaired interaction between XPG and TFIIH (34). We examined whether the XPG spacer deletion mutants are able to interact with TFIIH by co-immunoprecipitation experiments. Protein extracts from XP20BE cells transduced with XPG in wild-type form or with deletions in the spacer region were immunoprecipitated by using the monoclonal antibody 8H7 that recognizes the protein near the C terminus of XPG outside of the spacer region. The precipitated fraction was analyzed for the presence of XPG and TFIIH (XPB) by using appropriate antibodies, and the amount of co-precipitated XPB protein was quantified from three independent experiments (Fig. 6).

As expected, although no signal for XPB was observed in the immunoprecipitates of untransduced cells (Fig. 6) or cells transduced with the GFP control vector (data not shown), XPB co-purified with the wild-type XPG protein (Fig. 6). In contrast, the interactions between XPB and the \(\Delta 184\)–210 and \(\Delta 554\)–730 XPG spacer deletion mutant were reduced by 42 and 54\% respectively (Fig. 6, lanes 2 and 3), whereas the interaction with TFIIH was completely abolished with both XPG spacer deletion mutants \(\Delta 111\)–550 and \(\Delta 111\)–730 (Fig. 6, lanes 4 and 5). The strength of these interactions between XPG and TFIIH is thus consistent with the results of \textit{in vitro} and \textit{in vivo} NER activities as well as the recruitment of truncated XPG proteins to sites of UV damage. Apparently there is a correlation between the strength of the XPG-TFIIH interaction and NER activity.
**DISCUSSION**

**XPG Is a Modular Protein**—The XPG protein contains a 600-amino acid spacer region that is unique within the FEN-1 family of endonucleases and does not bear resemblance to any known protein. We evaluated how this region contributes to the substrate specificity and NER activity of XPG by generating and analyzing mutants of XPG with deletions in the spacer region. XPG is unique among nucleases of the FEN-1 family in that it cleaves bubble substrates, which do not contain free 5' ends, in addition to spliced arm and flap substrates. We found that nuclease activity on the spliced arm substrate was intact in XPG mutant proteins lacking the spacer region (Fig. 2A), in agreement with similar studies carried out with the yeast homolog Rad2 (55). These observations suggest that XPG is a modular protein, in which the spacer region is dispensable for the cleavage of substrates with ss/dsDNA junctions. Most interestingly, Δ111–550 and Δ111–730, and to a lesser degree Δ554–730, displayed reduced activity on bubble substrates (Fig. 2B), implying that residues within the region 111–550 are important for the substrate specificity of XPG. The observation that all of our mutants specifically bound to the bubble substrate with efficiencies comparable with the wild-type protein (data not shown) suggests that the spacer region does not contribute to recognition of the ss/dsDNA junction per se.

We have shown previously that XPG displays distinct requirements for substrate binding and cleavage (41). It is likely that the spacer region helps to position the protein on the substrate to form a catalytically competent complex following DNA binding. In line with this speculation, it has been shown that the flexible loop between the nuclease domains in FEN-1 assumes an ordered conformation upon substrate binding, which might be important for activation of the nuclease activity by properly aligning the enzyme with the substrate (30). Because all of the XPG spacer mutant proteins displayed additional insufficiencies in NER, our studies did not reveal how important the contribution of the spacer region to substrate specificity is in the context of the NER incision reaction. Because it has been shown that XPG has structural as well as catalytic roles in NER, it is tempting to speculate that the spacer region has a role in mediating a conformational change in XPG to activate its endonuclease activity only when XPG has been properly positioned in the NER pre-incision complex (56).

**The Spacer Region Mediates Interactions with TFIIH**—Previous studies have demonstrated that TFIIH interacts tightly with XPG and that TFIIH is the factor required for recruiting XPG to NER pre-incision complexes (13, 15, 32, 33). Two regions in XPG have been reported to be responsible for mediating the interaction with TFIIH by co-immunoprecipitation of in vitro translated proteins. Region 1–377 has been shown to interact with XPB and XPD and region 747–928 with XPB, XPD, p62, and p44 (33), suggesting that there are multiple interaction sites between XPG and TFIIH. Our studies indicate that residues within the region 111–550 of XPG are crucial because their deletion completely abolishes NER activity as well as interaction with TFIIH. This may be due to the loss of some key amino acids and/or altered XPG conformation. These results do not imply that other regions of XPG are not involved in interactions with TFIIH. Indeed, deletion of residues 554–730 of XPG reduces the affinity toward TFIIH by about 50%. Sequence comparisons of XPG homologs revealed two conserved domains that are only present in higher eukaryotes termed D1 (residues 184–210) and D2 boxes (residues 890–984), respectively (51). Our studies reveal that deletion of the D1 box from XPG reduces the affinity of XPG for TFIIH by about 40% and leads to a reduction in NER activity. The role of the D2 box has not yet been investigated, but it is tempting to speculate that it also contributes to NER activity by interacting with TFIIH, as the region 747–928 has been shown to interact with multiple subunits of TFIIH (33).

A recent study has shown that the p62 subunit of TFIIH contains a pleckstrin/phosphotyrosine homology domain of 108 amino acids that specifically interacts with XPG (57). This was identified as a discrete structural domain by limited proteolysis experiments and might have been missed by sequence-based predictions. It will be of interest to determine which part of XPG is responsible for mediating the interaction with p62 as well as mapping the remaining XPG-TFIIH interaction sites in more detail.

XPG region 668–747 has been reported to interact with RPA (35), and we were interested in finding out whether deletion of that region would interfere with the interaction with RPA. Unfortunately, we were unable to detect an interaction between XPG and RPA in XP-G/CS cells transduced with wild-type or mutant XPG with the co-immunoprecipitation conditions that were used to investigate the XPG-TFIIH interaction (data not shown). We therefore are unable to make a definite statement on whether the XPG spacer region is involved in an interaction with RPA.

**The TFIIH-XPG Interaction Is Required for Stable XPG Recruitment as Well as Subsequent Steps in the NER Pathway**—In a recent study, we investigated the properties of an XPG protein missing residues 225–231 in the spacer region; this protein is generated by alternative splicing in an unusual XP-G/CS patient (34). Although these residues are very close to the 184–210 D1 box deletion reported here, there are some striking differences between the properties of the two mutant XPG proteins. XPGA184–210 shows only a minor impairment in the interaction with TFIIH, significantly restores UV resistance in XP-G/CS cells, retains weak NER activity but is not visible in foci of UV damage. On the other hand, XPGA225–231 barely interacts with TFIIH, does not restore UV resistance, has no detectable NER activity but is found in foci. How might we explain these differences? The most likely explanation is that amino acids 184–210 contribute to an interaction with TFIIH that is required for the recruitment of XPG to the pre-incision complex, leading to slower kinetics of recruitment and repair. On the other hand, residues 225–231 appear to play a role in stabilizing an interaction that is required in a subsequent step of the NER reaction. The loss of this interaction with TFIIH apparently precludes NER from occurring altogether. These observations support the notion that XPG is a modular protein, in which separate domains fulfill various functions required for NER.

**The Involvement of XPG in Progression through the NER Pathway**—There is increasing evidence that the sequential assembly of the six main repair factors, XPC-HR23B, TFIIH, XPA, RPA, XPG, and ERCC1-XPF, mediates the NER incision reaction (13, 19, 32, 58). This model requires that mechanisms are in place to facilitate the transition from one reaction intermediate to the next. Three events can be invoked to ensure progression through the NER pathway. First is ATP hydrolysis. The energy-dependent helix opening by the XBP and XPD helicases is responsible for an irreversible structural change of intermediates that facilitate recruitment of factors acting downstream of TFIIH (16, 18, 32, 59). Second, a factor that could contribute to pathway progression is the post-translational modification of one of the proteins involved in the pathway, leading to a conformational change that facilitates the transition from one intermediate to the next. It has been shown that NER activity is regulated by post-translational modifications, but if and how this might contribute to pathway progression is not yet clear (60–63). Third, the nature of the interac-
tion between NER factors and their substrates is crucial. To ensure smooth transition between reaction intermediates, multiple relatively weak and transient interactions are employed instead of strong and irreversible binding interactions (64). We suggest that XPG contributes to the progression through the NER pathway in this way with its independent domains that participate in the interactions with other NER protein and DNA substrates. The observation that XPG has distinct requirements for binding and catalysis could provide an element of irreversibility to this process in that the endonuclease activity is only activated once a proper conformation has been reached (41). We believe that the spacer region of XPG, and in particular the N-terminal part of the spacer, is crucial for progression through the NER pathway as it mediates multiple interaction points with other proteins and controls the efficiency of the endonuclease activity of XPG on substrates that mimic NER intermediates.

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