Open complex formation around a lesion during nucleotide excision repair provides a structure for cleavage by human XPG protein

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Human XPG nuclease makes the 3' incision during nucleotide excision repair of DNA. The enzyme cleaves model DNA bubble structures specifically near the junction of unpaired DNA with a duplex region. It is not yet known, however, whether an unpaired structure is an intermediate during actual DNA repair. We find here that XPG requires opening of >5 bp for efficient cleavage. To seek direct evidence for formation of an open structure around a lesion in DNA during a nucleotide excision repair reaction *in vitro*, KMnO₄ footprinting experiments were performed on a damaged DNA molecule bearing a uniquely placed cisplatin adduct. An unwound open complex spanning ~25 nucleotides was observed that extended to the positions of 5' and 3' incision sites and was dependent on XPA protein and on ATP. Opening during repair occurred prior to strand incision by XPG.

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Introduction

Nucleotide excision repair (NER) is a major pathway that removes UV and chemically induced damage from DNA. The biochemical steps in the repair of damaged bases include damage recognition, formation of incisions ~26–29 nucleotides (nt) apart on each side of a lesion, removal of the damage as part of an oligonucleotide and filling of the gap by DNA synthesis and ligation to restore the original duplex. In eukaryotes, the two incisions made during NER are catalysed by separate DNA endonucleases. The XPG protein and the ERCC1–XPF complex make the 3' and 5' incisions respectively in humans (O'Donovan *et al.*, 1994a; Mu *et al.*, 1996; Sijbers *et al.*, 1996). Both of these nucleases have remarkable structure-specific properties. XPG and its *Saccharomyces cerevisiae* homolog Rad2 cleave DNA structures specifically near the junction of unpaired and duplex DNA, cutting the strand in which the unpaired region has a polarity moving 3'→5' away from the junction (Harrington and Lieber, 1994; O'Donovan *et al.*, 1994a; Habraken *et al.*, 1995). Conversely, the ERCC1–XPF nuclease (and budding yeast counterpart Rad10–Rad1) cleaves such structures with the opposite polarity, on the unpaired strand that passes 5'→3' away from a junction (Bardwell *et al.*, 1994; Matsunaga *et al.*, 1996; Sijbers *et al.*, 1996).

The properties of these two groups of nucleases have suggested a plausible model for NER in eukaryotes, in which the action of repair proteins generates an open, bubble or flap-like structure which is the substrate for endonucleolytic incision on each side of the lesion (Bardwell *et al.*, 1994; O'Donovan *et al.*, 1994a; Friedberg *et al.*, 1995). To search for evidence for or against this model, we have now examined the requirements for incision by XPG on various model DNA substrates and precisely determined the sites of cleavage by XPG at different double-strand/single-strand junctions. We then sought direct evidence for DNA opening during NER of a uniquely placed cisplatin adduct. Sites sensitive to KMnO₄ modification were formed around the lesion during repair, demonstrating that prior to strand scission, there is local opening around DNA damage that extends to the positions of the dual incision sites. This provides strong evidence for formation of an open complex intermediate during mammalian NER.

Results

Cleavage of model structures by XPG

Recombinant XPG protein was produced in an insect cell system and purified (Figure 1A). Initial studies used the DNA structure shown in Figure 1B, which contains a centrally unpaired bubble of 30 nt, flanked by two duplex regions of 30 bp. One strand was 5'-labelled with ³²P by polynucleotide kinase. Treatment with S1 nuclease confirmed that only the central segment was unpaired under our reaction conditions (Figure 1C). When incubated with XPG protein, the labelled strand was specifically cut on the 3'-side of the unpaired region. In this reaction, ~55% of the input substrate was cleaved in 90 min at 37°C. A divalent cation was required for the reaction and either Mg²⁺ or Mn²⁺ served as a cofactor over a broad concentration range, but neither Ca²⁺ nor Zn²⁺ could substitute (Figure 1C and data not shown). Maxam–Gilbert sequencing ladders were used to determine the exact sites of scission on each strand (Figure 1C and data not shown). The principal sites of cleavage were 0, 1 and 2 nt away from the single-strand/duplex junction. The different local sequences in each case (Figure 1B) confirm that XPG acts as a structure-specific, rather than a sequence-specific, endonuclease.

XPG can also cleave splayed arm structures near the single-strand/duplex DNA junction (O'Donovan *et al.*, 1994a; Cloud *et al.*, 1995). We investigated XPG cleavage of a splayed arm substrate (Y in Figure 2A) and found cutting near the single-strand/duplex region as expected, but also several shorter products resulting from cleavage in the unmatched 5' arm (Figure 2B). No internal cleavage occurred when the strand was fully paired (DS in Figure 2C). A different array of products was released

Fig. 1. XPG nuclease cleaves a synthetic bubble substrate at the double-strand/single-strand junction. (A) Purified recombinant human XPG protein (400 ng) was applied to a 10% SDS–polyacrylamide gel and stained with Coomassie Blue. The molecular masses of protein standards are indicated. (B) The structure of bubble substrate BS-A bearing a 30 nt unpaired region. The positions of cleavage by XPG protein on each strand are indicated by arrows. (C) Substrate BS-A was 5'-end-labelled on the right strand, purified and incubated for 90 min with the following variations on standard reaction conditions (displayed from left to right): 40 ng (300 fmol) XPG with CaCl_2 at 10 or 5 mM, with MnCl_2 or MgCl_2 at 10, 5, 2 or 1 mM, with no divalent cation (–), no XPG (–) or S1 nuclease (0.05 or 0.1 U). The products were analysed on a denaturing 12% polyacrylamide gel. Maxam–Gilbert G+A and C+T sequencing ladders of the labelled strand are displayed as markers.

when the same labelled strand was completely unpaired (compare Y, lane 2, and SS, lane 3, in Figure 2B). These internal products were presumably due to characteristic secondary conformations adopted by the single-stranded regions (Gold *et al.*, 1995) which generate structural substrates for XPG. A similar cleavage at secondary structures in unpaired arms was observed for the 5' nuclease activity of *Taq* polymerase and could be eliminated at high temperature (Lyamichev *et al.*, 1993). The known cleavage of single-stranded M13 DNA by XPG (O'Donovan *et al.*, 1994b) seems very likely to occur at such secondary structures.

With all of these 5'-labelled substrates, a small product was released that co-migrated with the product of treatment with S1 and mung bean single-strand-specific endo-

nucleases (S1 and M in Figure 2C) and was presumed to be mononucleotide. To verify this, the products of XPG cleavage of a bubble substrate were compared with markers. In addition to the bubble-specific cleavage product (near the top of the gel under these conditions), the small product migrated exactly with the mononucleotide (dCMP) marker (Figure 2D). Both the structure-specific cleavage and mononucleotide release are intrinsic activities of XPG, as demonstrated by gel filtration chromatography in 0.2 (Figure 2E) or 1 M KCl (Figure 2F). The relative release of 5'-mononucleotide was usually minor but was influenced by ionic conditions and elevated in higher concentrations of Mg^{2+} , Mn^{2+} or KCl (data not shown). This may reflect different conformations taken by the substrate under varying salt conditions. With the conditions used in Figure 2D, 39%

Fig. 2. XPG nuclease cleaves single-stranded DNA in a structure-dependent manner and releases 5'-mononucleotide. **(A)** Single-stranded (SS), duplex (DS) and splayed arm (Y) substrates. Positions of cleavage by XPG protein on the 5'-end-labelled strand (asterisk) are indicated by arrows. **(B)** Y substrate (lanes 1–2 and 6) or SS substrate (lane 3) was 5'-end-labelled on the strand indicated in (A) and incubated under standard reaction conditions with no enzyme (lane 1), 20 ng XPG protein (lanes 2 and 3) or 1 U S1 nuclease (lane 6). Maxam–Gilbert sequencing ladders of the labelled strand are displayed in lanes 4 and 5. **(C)** Y substrate (lanes 1–4) or DS substrate (lanes 5–7) was 5'-end-labelled on the strand indicated in (A) and incubated under standard reaction conditions with no enzyme (lane 5) or with 20 ng XPG protein (lanes 1 and 6), 80 ng XPG (lane 7) or 1 U S1 nuclease (lane 2), mung bean nuclease (M, lane 3) or calf intestinal alkaline phosphatase (C, lane 4). **(D)** Bubble substrate BS-A (Figure 1A) was labelled on the left strand and incubated without enzyme (lane 1) or with 40 ng XPG (lane 2) or 1 U CIP (lane 3) under standard reaction conditions. Reaction products were analysed on a denaturing 20% polyacrylamide gel. Marker oligonucleotides representing the five 5'-terminal residues of the labelled strand are shown: lane 4, labelled dCMP; lanes 5–8, 5'-end-labelled dCC, dCCA, dCCAG and dCCAGT. Maxam–Gilbert G+A (lane 9) and C+T (lane 10) reactions are shown. **(E)** Bubble substrate BS-A (left strand labelled) was incubated in standard reactions containing no XPG (lane 19), 40 ng XPG (lane 18), 40 ng XPG diluted in column running buffer (lane 17) or 1 µl peak XPG-containing fractions eluted from a Superose 12 column in buffer containing 0.2 M KCl. An autoradiograph of a 15% denaturing polyacrylamide gel is shown. Maxam–Gilbert sequencing ladders (lanes 1 and 2) and oligonucleotide size markers (lanes 3–6) are the same as shown in (D). **(F)** Graph depicting the results of Superose 12 gel filtration analysis of XPG carried out in buffer containing 1 M KCl. Peak XPG-containing samples were analysed on a 15% sequencing gel, amounts of the 61-mer and mononucleotide products were quantified with a PhosphorImager and presented as a fraction of the total signal for each reaction.

of 5'-label was found in uncleaved material, 55% in product resulting from cleavage at the single-strand/duplex junction, 6% in mononucleotide and a trace amount (<1%) of material co-migrating with 4–5-mer.

Requirements for bubble cleavage by XPG

XPG acts principally as a structure-specific endonuclease *in vitro* and results such as those in Figure 1 suggest that during NER the enzyme makes the 3' incision at a single-

Fig. 3. The requirements for bubble cleavage by XPG. **(A)** Substrates A–E represent annealed 90-mer oligonucleotides bearing a central, unpaired bubble region of 30 (BS-A), 20 (BS-B), 15 (BS-C), 10 (BS-D) or 5 (BS-E) nt. Substrates F–J represent annealed oligomers bearing a 30 nt bubble and one flanking arm of 30 bp with the opposite arm of 25 (BS-F), 20 (BS-G) or 15 (BS-H) nt. Each substrate was 5'-end-labelled on the top strand. **(B)** Bubble substrates A–E were tested in a time course study under standard conditions. An autoradiogram of the denaturing 12% polyacrylamide gel is shown. Lanes labelled G+A and C+T represent Maxam–Gilbert sequencing markers for the series of reactions displayed to the left. **(C)** Quantification of the experiment shown in (B). Phosphorimager data are expressed as a percentage of the total product of cleavage at the double-strand/single-strand junction. **(D)** Bubble substrates A, F, G and H were tested in a time course study under standard conditions and analysed on a 12% gel. Maxam–Gilbert sequencing reactions of the labelled strand correspond to the series of reactions displayed to the left.

strand/double-strand junction in some open or bubble-like reaction intermediate. To test parameters important for bubble cleavage, the substrates shown in Figure 3A were

constructed. Structures A–E comprise a series of structures formed by annealed partially complementary 90-mers that bear opposing C and T tracts and have bubbles of

decreasing size (30, 20, 15, 10 and 5 nt). Shortening of the bubble was matched by the insertion of sequence in the flanking duplex arms. This ensured that the overall substrate size, the sequence context of each junction and each double-strand terminus remained identical. This series of structures was tested for cleavage by XPG in a time course study. Analysis of the products in conjunction with Maxam–Gilbert sequencing markers (Figure 3B) showed that the same site relative to the bubble was cleaved in

each case, regardless of bubble size (see top strand, Figure 1B). Quantification of product release (Figure 3C) showed that bubbles ranging from 10 to 30 nt were comparable substrates for XPG, but that the 5 nt bubble was a poor substrate, with <5% cleaved after 90 min incubation. We also asked whether the length of duplex sequence flanking a site of cleavage influenced the reaction by testing substrates A, F, G and H (Figure 3A). These otherwise identical 30 nt bubbles have one constant double-strand arm of 30 bp, while the arm nearest the cleavage site decreases from 30 to 25 to 20 to 15 bp. Reducing arm length in this way had little effect on the reaction (Figure 3D).

On these model structures, structure-specific endonucleolytic activity is intrinsic to purified XPG protein and does not require other protein factors. However, during repair XPG operates within the context of interactions with other proteins that may modulate or help target endonuclease activity. Two candidate factors are RPA and PCNA. RPA is a single-stranded DNA binding protein required for the incision step of NER (Coverley *et al.*, 1992; Shivji *et al.*, 1992; Aboussekhra *et al.*, 1995; Mu *et al.*, 1995). RPA interacts with both XPG and XPA (He *et al.*, 1995) and under some conditions RPA can unwind DNA in an ATP-independent fashion (Georgaki *et al.*, 1992). Because the bubble with an unpaired region of 5 nt is cleaved very weakly compared with bubbles of 10 nt or larger (Figure 3), we speculated that RPA might help open up the region further and increase cleavage of this structure. However, no stimulation of XPG cleavage by recombinant human RPA on either the 30 or 5 nt bubbles was observed (Figure 4A, lanes 6–8 and 15–17). In fact, increasing amounts of RPA inhibited nuclease activity on the 30 nt bubble (Figure 4B). RPA has a binding site size on DNA of between 8 and 30 nt (Kim *et al.*, 1992; Blackwell and Borowiec, 1994) and may be unable to bind a bubble of 5 nt. In any case, the positions of XPG incisions were unchanged in the presence of RPA.

It has recently been shown that PCNA physically interacts with an enzyme related to XPG, FEN-1 (DNase IV), and stimulates the structure-specific nuclease activity of the latter protein (Wu *et al.*, 1996). Because PCNA is involved in NER, we wondered whether it might also affect XPG activity. However, we found no effect of adding recombinant human PCNA to our reactions (Figure 4A, lanes 3–5, 12–14 and panel B).

Open intermediates during NER

The properties of XPG described above suggest a plausible model for part of the NER reaction mechanism. During

Fig. 4. The effect of RPA and PCNA on bubble cleavage. (A) Bubble substrates BS-A and BS-E depicted in Figure 3A were labelled on the bottom strand and tested in standard reactions containing no XPG (lanes 1 and 10) or 40 ng (15 nM) XPG (lanes 2–8 and 11–17). PCNA was added at 1.7, 3.4 or 13.6 nM (lanes 2–4 and 11–13). RPA was added at 1.6, 3.1 or 12.6 nM (lanes 6–8 and 14–16). Autoradiographs of denaturing 20% polyacrylamide gels are shown with Maxam–Gilbert C+T (lanes 9 and 18) and G+A (lane 19) sequencing ladder of the labelled strand in each case as size markers. (B) Quantification of PhosphorImager data for the experiment shown in (A) and a separate experiment using substrate BS-A labelled on the top strand (not shown), with RPA (top graph) or PCNA (bottom graph) added to 3.4, 13 or 52 nM as indicated. Data are expressed as the bubble cleavage product or mononucleotide product normalized to the total radioactivity in each reaction.

Single Lesion Substrate:

Footprinting reactions:

Fig. 5. Procedure for KMnO_4 footprinting experiments. Closed circular Pt-GTG DNA was cleaved at a unique *AvaII* site, radiolabelled on the 3'- or 5'-end and then used in footprinting reactions as indicated.

repair, a sufficiently opened DNA structure may be actively formed around a lesion and present a substrate for XPG cleavage on the 3'-side of DNA damage (and ERCC1-XPF on the 5'-side). To find out whether such an open structure can be detected during an NER reaction, KMnO_4 was used as a probe for single-stranded character (Borowiec *et al.*, 1987; Jiang and Gralla, 1995). KMnO_4 oxidizes and degrades thymine residues, producing sites in the phosphodiester backbone of DNA that are subject to subsequent cleavage in hot alkali. In B-DNA, thymines are shielded by base-stacking interactions, but unwinding of the DNA considerably increases the sensitivity of T residues to oxidative attack. Consequently, strand breaks that form at positions of T residues following KMnO_4 and piperidine treatment are markers of open DNA regions. The experimental design is summarized in Figure 5. Whole cell extracts were incubated under repair conditions and a linear DNA substrate bearing a single, precisely placed cisplatin lesion was added. DNA was labelled at a restriction site 140 bp 3' of the lesion on either the damaged or the non-damaged strand. At various times during repair, KMnO_4 was added to 6 mM for 1 min, reactions were quenched with reducing agent and the purified DNA was treated with piperidine and analysed on sequencing gels.

Some of the cell extracts used in the following experiments are characterized for activity in Figure 6. Repair was monitored in a Southern hybridization assay which measures the release of platinated oligonucleotide as a consequence of incision on the 3'- and 5'-sides of the Pt-

Fig. 6. Dual incision assay showing the ATP and XPA dependencies of NER in extracts used in footprinting experiments. Unlabelled Pt-GTG DNA was incubated in repair buffer (–dNTPs) containing ATP (lanes 1, 3 and 4) or lacking ATP (lanes 2, 5 and 6) with 100 μg HeLa extract protein (lanes 1 and 2) or 200 μg GM2345 extract protein in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of 80 ng XPA protein. After 15 min, DNA was purified, cleaved with *HindIII* and *XhoI* and analysed. DNA on a filter was hybridized with a radiolabelled oligonucleotide probe designed to detect the excision of platinated products and formation of uncoupled incisions as described (Sijbers *et al.*, 1996). Lane 7 displays a platinated 24-mer as a size marker.

GTG lesion (Moggs *et al.*, 1996). Incubation of Pt-GTG with repair-proficient HeLa cell extract led to the release of platinated 24–30-mer oligonucleotides (Figure 6, lane 1). This assay also detects uncoupled 3' incisions (3' incisions without 5' incisions; see Sijbers *et al.*, 1996). The dual incision reaction was ATP dependent (Figure 6, lane 2), as previously noted (Wood *et al.*, 1988; Calsou and Salles, 1994; Mu *et al.*, 1996). Extract from an NER-deficient XP group A cell line showed no dual incision (lane 3) and activity could be restored to the XP-A extract by adding purified XPA protein to the reaction mixture (lane 4). This complemented reaction was also ATP dependent (lanes 5 and 6).

Formation of sensitive sites on the damaged strand

For the adduct used here, the main incision formed by XPG falls nine phosphodiester bonds 3' of the intrastrand platinum crosslink (Moggs *et al.*, 1996). In conjunction with DNA sequencing markers, this 3' incision serves as a point of orientation for the positions of the platinum crosslink and 5' incision, indicated in Figure 7A. For reference, bases on the damaged strand are numbered according to their positions relative to the central T (designated zero) in the crosslinked GTG sequence. Bands corresponding to 3' incisions were seen when Pt-GTG DNA was labelled on the damaged strand and incubated

Fig. 7. Opening of DNA around a Pt–GTG crosslink during repair. **(A)** Autoradiograph of a 6% polyacrylamide gel. Pt–GTG DNA (3'-labelled on the damaged strand) was incubated in repair buffer containing ATP (–dNTPs) or lacking ATP (–dNTPs) as indicated, with 100 µg HeLa cell extract protein, 200 µg XPA-defective GM2345 extract protein or 100 µg BSA as indicated. GM2345 reactions in lanes 4 and 8 were complemented by adding 80 ng purified XPA protein. After 15 min, reactions in lanes 2–4 and 6–10 were treated with KMnO_4 . The actual sequence of the relevant portion of the damaged strand is displayed above. Residues 3' of T[0] are denoted by positive integers [+*n*], with the principal 3' incision falling between C[+9] and T[+10]. Residues 5' of T[0] are denoted by negative integers [–*n*], with the main 5' incision falling between C[–17] and C[–16]. Additional DNA markers were made by dideoxy sequencing of an unrelated single-stranded M13mp18 DNA sequence and displayed as G, A, T and C ladders for size orientation. **(B)** 3'-end-labelled Con-GTG DNA (lanes 1–5) or Pt–GTG DNA (lanes 6–10) was incubated in repair buffer (+ATP, +dNTPs) containing 100 µg HeLa extract protein or 100 µg BSA (B) as indicated. Reactions were terminated after the time of incubation shown.

with HeLa extract under repair conditions (Figure 7A, lane 1). In this experiment, dNTPs were omitted so that repair DNA synthesis was inhibited, trapping 3' incised intermediates. Some of these are slightly degraded to form smaller labelled species, as previously observed (Moggs *et al.*, 1996).

When KMnO_4 was added to the HeLa extract reaction after 15 min repair, an array of T residues flanking the Pt-GTG was susceptible to cleavage, indicating the presence of single-stranded character around the DNA lesion (Figure 7A, lane 2). The KMnO_4 sensitivity of four thymines (T[0], T[-2], T[-4] and T[-5]) was partially due to the structure of the Pt-GTG lesion itself, since they reacted in the absence of extract (Figure 7A, lanes 9 and 10). In the presence of cell extract, however, T[-2] became markedly more sensitive to oxidation (compare lanes 6-8 with 9-10). Additionally, HeLa extract induced sensitivity of additional T residues flanking the lesion on the 3'-side (T[+5]) and on the 5'-side (T[-7], T[-8] and T[-10]), as shown in lane 2. None of these cleavage sites was observed in the same DNA sequence without a lesion (Figure 7B, lanes 1-5).

Hypersensitivity of T residues [+5], [-7], [-8] and [-10] was weakly evident in the minutes immediately following the addition of labelled DNA to the extract (Figure 7B, lane 7), but was strong after 15 min incubation (lane 8) and less pronounced after 45 min (lane 9). This is consistent with the previously reported time course for the repair of this GTG adduct, where little reaction was observed after 5 min and most repair took place during the next 15 min (Moggs *et al.*, 1996).

The positions of the sensitive T residues detected by this assay, mapped in comparison with DNA size markers, are indicated above Figure 7A. T residues beyond the 3' and 5' incisions positions were not reactive, indicating that the area of unwinding does not extend significantly beyond the borders of the repair patch. The site of the 3' incision lies between the platinum lesion and the 3'-end-label on the damaged strand and so the KMnO_4 -sensitive sites detected in these experiments had occurred on molecules that had not yet been 3' incised. This indicates that open complex formation precedes incision formation. An identical pattern of sensitivity to oxidation by KMnO_4 was

Fig. 8. Analysis of the non-damaged strand and XPG-independence of opening. (A) Pt-GTG DNA 5'-end-labelled on the non-damaged strand was used in reactions that were otherwise identical to those described in Figure 7A, lanes 1–9. The sequence of the relevant portion of the non-damaged strand is shown. Residues on the non-damaged strand are numbered in a way similar to Figure 7, but designated as prime, T'[n], to distinguish them from their complement (with A'[0] lying opposite T[0]). (B) Pt-GTG DNA 3'-end-labelled on the damaged strand was incubated in repair buffer (+ATP, +dNTPs) in the presence of 100 µg HeLa extract protein, 200 µg XPG-defective XPG83 extract protein or 100 µg BSA (B) as indicated. Reactions in lanes 4, 5, 7 and 8 were complemented with 50 ng purified recombinant XPG protein. Lanes 9 and 10 also contained 1 µl neutralizing polyclonal anti-XPG antiserum or non-neutralizing anti-XPG monoclonal antibody 8H7 respectively. Reactions were treated with KMnO₄ at the times indicated (min). An autoradiograph of a 6% sequencing gel is shown.

observed in experiments lacking (Figure 7A) or containing (Figures 7B and 8A) deoxyribonucleotides.

Opening around the lesion requires XPA and ATP

To verify that the opening of DNA around the platinum lesion in the extracts was mechanistically related to NER, reactions were performed with extracts from a repair-deficient XP-A cell line (GM2345) that is defective in the essential NER protein XPA. The 3'-end-labelled Pt-GTG substrate was incubated with GM2345 whole cell extract and treated with KMnO₄ after 15 min. The pattern of sensitive bands (Figure 7A, lane 8) included the three sites (T[0], T[–4] and T[–5]) that show moderate sensitivity in the absence of extract (see Figure 7A, lanes 9 and 10), although the presence of GM2345 extract slightly increased their reactivity. The T[–2] site, which is only weakly sensitive in the absence of extract, became hypersensitive in the presence of GM2345 extract, reaching the intensity of its three neighbours (Figure 7A, compare lanes 3 and 8 with 9 and 10). This indicates that XPA function is not required for the slight hypersensitivity generated in extracts

immediately surrounding the platinum lesion (involving T[–2]). Addition of XPA protein to reactions with GM2345 extract restored incision function (Figure 6) and generated a full pattern of KMnO₄-sensitive sites identical to that produced by HeLa extract (Figure 7A, lane 4). The extensive opening up of DNA away from the lesion towards each of the incision sites (involving residues T[+5], T[–4], T[–5] and T[–7]) is therefore strongly dependent on XPA.

The overall incision reaction is strictly ATP dependent (Figure 6) and requires TFIIH, which contains ATP-dependent DNA helicase activities (Schaeffer *et al.*, 1993; Hoeijmakers *et al.*, 1996). We asked whether some or all of the observed unwinding was also dependent on ATP. The extract-independent sites (T[0], T[–4] and T[–5]) and the hypersensitive site at T[–2] still occurred in the absence of ATP (Figure 7A, lanes 6 and 8). However, in the absence of ATP, no 3' incision was detectable and all other flanking T residues remained insensitive to KMnO₄ in both HeLa extract and GM2345 extract with or without XPA protein (Figure 7A, lanes 5–8).

Fig. 9. A model for open complex formation around a 1,3-intrastrand d(GpTpG)-cisplatin crosslink during NER. Results of the experiments shown in Figures 7 and 8 are depicted relative to the sequence surrounding the Pt-GTG lesion. Residues on the damaged strand are numbered. Opening of the helix, as evidenced by reactivity to KMnO_4 of circled and boxed T residues, is depicted by placement of residues above or below the axis of the duplex sequence. The positions of P1 nuclease-sensitive sites (P1^*) are shown. Locations of the dual incisions for this lesion (Moggs *et al.*, 1996) are indicated by arrows.

Formation of sensitive sites on the non-damaged strand

A comparable analysis was performed on the non-damaged strand. In the region opposite the lesion this strand contains fewer T residues that can be monitored by permanganate sensitivity (Figure 8A, top). Residue $\text{T}'[+3]$ was mildly sensitive to oxidation in the absence of extract (Figure 8A, lane 9), slightly more reactive in HeLa extract without ATP and even more reactive in the presence of ATP (lanes 1–2 and 5–6). Moreover, the more distant residues $\text{T}'[-15]$ and $\text{T}'[-18]$ became sensitive in the presence of HeLa extract and ATP at 15 min. The sensitivity of the latter residues was not evident after only 1 min incubation in HeLa extract (data not shown). The sensitive sites at $\text{T}'[-15]$ and $\text{T}'[-18]$ did not occur in GM2345 extract alone, but appeared when the extract was complemented by the addition of XPA protein and depended on the presence of ATP (Figure 8A, lanes 3–4 and 7–8). No other T residue was found to be hypersensitive to oxidation.

As an alternative means of probing for single-stranded character, the single-strand-specific P1 nuclease was added to repair reactions. Three XPA-dependent nuclease hypersensitive sites were formed on the damaged strand just 5' of the Pt lesion during ongoing NER and several sites appeared on the non-damaged strand (data not shown). These P1-sensitive sites are shown in Figure 9 and correspond to the strongest region of opening detected by KMnO_4 . As suggested previously, KMnO_4 appears to

provide a smaller and more sensitive probe for DNA opening than endonucleases (Borowiec *et al.*, 1987).

XPG function is not required for DNA unwinding around a lesion

The above experiments provide evidence for local opening up of the DNA around the site of DNA damage during NER, spanning a region of ~25 bp that lies within the domain defined by the 3' and 5' incisions (summarized in Figure 9). This supports the idea that an open structure is indeed the substrate for XPG action during NER and it was of interest to know if the XPG nuclease itself was required for opening to occur. This was tested in two ways. Figure 8B shows the results of incubating 3'-end-labelled Pt-GTG in HeLa extract (lanes 1–2) or in an XPG-defective extract, XPG83. Extracts from the latter cell line are defective in the formation of 3' incisions, but repair can be restored by the addition of purified XPG protein (Sijbers *et al.*, 1996). Complementation of XP lines sometimes produces a weaker repair signal than that observed in HeLa cells (see for example Figure 6) and so the numbers of transient 3' incision intermediates in reactions with XPG83 extract and XPG protein were barely detectable in the 3'-end-labelling assay (Figure 8B, lane 3–5). However, addition of KMnO_4 to the reaction at 15 min either in the absence or presence of purified XPG led to a pattern of sensitivity that was essentially identical to that formed in HeLa extract, suggesting that

structural opening occurs equally well in the absence of XPG activity (Figure 8B, compare lanes 6 and 7 with 2 and 11). Likewise, addition of a polyclonal antiserum which inhibits XPG nuclease (O'Donovan *et al.*, 1994a) and dual incision activity during NER (J.G.Moggs and R.D.Wood, unpublished data) inhibited the formation of 3' incisions in the HeLa extract, but did not reduce or alter the pattern of KMnO₄ sensitivity on the damaged strand (Figure 8B, lane 9) or the non-damaged strand (data not shown). A different, non-neutralizing anti-XPG antibody also had no effect on opening (lane 10). Together these results show that XPG function is not required for opening of the DNA surrounding the lesion during NER.

Discussion

Requirements for XPG cleavage

XPG is a member of a family of eukaryotic nucleases comprising two subfamilies, NER enzymes of ~1200 amino acids (vertebrate XPG, *S.cerevisiae* Rad2 and *Schizosaccharomyces pombe* rad13) and replication-associated nucleases of ~500 residues (mammalian FEN-1/DNase IV, *S.cerevisiae* Rad27 and *S.pombe* rad2). Both subfamilies share a set of conserved domains that show homology to the 5'→3' exonuclease domain of eubacterial DNA polymerases (Carr *et al.*, 1993; MacInnes *et al.*, 1993; Scherly *et al.*, 1993; Harrington and Lieber, 1994; Robins *et al.*, 1994). The NER enzymes cleave bubbles, splayed arms, flaps and weakly release mononucleotide (Habraken *et al.*, 1994a, 1995; O'Donovan *et al.*, 1994a; Cloud *et al.*, 1995). The FEN-1 group shows a preference for flap structures and releases mononucleotide more avidly. It is unclear whether either group can be classified as exonucleases in the usual sense. Wu *et al.* (1996) presented evidence that the 5' exonuclease activity of FEN-1 at a nick may simply reflect structure-specific cleavage activity due to breathing of the 5'-end to form a 1 nt 'flap', an interpretation consistent with our data for XPG as well.

The results presented here map the exact sites of enzymatic scission by XPG on two novel double-strand/single-strand junctions within a bubble molecule, showing cleavage within the double-stranded region 0, 1 or 2 nt from the predicted junction. XPG could cut either strand of the bubble, but only on the side where the unpaired strand had polarity 3'→5' moving away from the bifurcation. Either Mg²⁺ or Mn²⁺ served as cofactor, although it was previously suggested that XPG was inactive in Mn²⁺ (Habraken *et al.*, 1994a,b). The cleavage of bubble substrates was intrinsic to the purified recombinant XPG protein and did not require other protein factors. However, an enzyme to substrate ratio of 19:1 was required to obtain 50% cleavage of a 30 nt bubble. Since XPG presumably acts as part of a multiprotein complex during repair, it seems likely that some of the other repair proteins modulate its activity. We found that the incision factor RPA inhibited cleavage of a 30 nt bubble by XPG, perhaps because the two proteins compete for binding to DNA. Similarly, Murante *et al.* (1995) found that *Escherichia coli* Ssb inhibited the cleavage of flap substrates by calf FEN-1. RPA may serve to protect the non-damaged strand from inappropriate scission by XPG during NER.

In contrast, Matsunaga *et al.* (1996) recently reported

that RPA stimulated cleavage of bubble substrates by purified XPG. The assay conditions were roughly similar to ours, but stretches of mixed heterologous sequence were used to form the single-stranded regions, rather than homopolymeric tracts. In the absence of RPA, our 30 nt bubble substrate was cleaved ~50-fold more efficiently than the 30 nt bubble substrate of Matsunaga *et al.* While the specific activities of the two XPG preparations might be different, we also suggest that the structure adopted by our substrates presents a more open configuration to the enzyme compared with mixed sequences, which can form various secondary structures. The interplay between XPG and RPA on model structures and on damaged substrates during repair remains an interesting area for further exploration.

PCNA is required for repair synthesis (Shivji *et al.*, 1992), but may also be present during the NER incision reaction. PCNA interacts with FEN-1 (Li, X. *et al.*, 1995; D.P.Lane, personal communication) and can stimulate FEN-1 nuclease on a flap substrate when present at a 12-fold molar excess (Wu *et al.*, 1996). PCNA did not stimulate XPG on our 30 nt bubble. Although the molar ratios of DNA to PCNA are similar in the two studies, the substrates and buffers used were different, as were the nuclease to DNA and nuclease to PCNA ratios. With these caveats in mind, our results are consistent with the suggestion by Wu *et al.* that the two nuclease subfamilies differ in their mode of substrate recognition. They proposed that the FEN-1 family of nucleases load via the single-strand arm of flaps, in a PCNA-stimulated or -stabilized manner. The NER nucleases, on the other hand, can bind and cleave bubble substrates that lack a free single-strand arm (and are refractory to FEN-1 cleavage), suggesting that they recognize their structural target by a different mechanism that is PCNA independent.

Open intermediates during NER

The properties of the eukaryotic NER nucleases have suggested the possibility that an open structure forms during repair and creates substrates for cleavage by structure-specific nucleases. Even though evidence for such opening has not yet been presented, the model can already be found in a textbook (Friedberg *et al.*, 1995). Determining whether or not this occurs is of fundamental importance for understanding the mechanism of the repair reaction. Using KMnO₄ footprinting, we observed opening of the DNA spanning an ~25 bp region around the site of a Pt-DNA lesion, within the area delineated by the dual incisions of repair. Similar techniques have been used widely to study DNA structural dynamics and have helped to elucidate open complex formation at origins of replication (Borowiec *et al.*, 1988; Ishimi and Matsumoto, 1994) and RNA polymerase II promoters (Jiang and Gralla, 1995). The considerable extent of hypersensitivity to KMnO₄ observed in our experiments reflects an opening and loss of base pairing that must be protein driven (Bowater *et al.*, 1994).

A model to explain the footprinting results is presented in Figure 9. We found that the platinum crosslink itself conferred permanganate sensitivity to several nearby residues in the absence of extract, consistent with evidence for structural distortions of the double helix by this and other lesions (Anin and Leng, 1990; Bellon *et al.*, 1991;

Schwartz and Leng, 1994; van Garderen and van Houte, 1994; Barone *et al.*, 1995; Kim and Choi, 1995). Further limited opening around the adduct was induced by addition of cell extract (even in the absence of ATP), as evidenced by the hypersensitivity of residue T[−2]. XPA protein binds preferentially to damaged DNA in an ATP-independent manner, suggesting that it (together with RPA) is involved in the damage recognition step of NER (Robins *et al.*, 1991; Jones and Wood, 1993; Asahina *et al.*, 1994; He *et al.*, 1995; L.Li *et al.*, 1995a; Matsuda *et al.*, 1995). We note, however, that even XPA-defective extracts conferred some hypersensitivity to residue T[−2], suggesting that proteins in addition to XPA play a role in damage recognition and binding.

Further opening away from the lesion and towards the two incision sites required both ATP and the XPA protein. The ATP requirement suggests the action of one or both of the DNA helicases XPB (3'→5') and XPD (5'→3'), which are needed for incision formation as part of the multisubunit TFIIH complex. There is evidence that XPA binds to the ERCC1–XPF nuclease complex (Park and Sancar, 1994; L.Li *et al.*, 1995b; Nagai *et al.*, 1995), as well as to TFIIH (Park *et al.*, 1995), suggesting the possibility that XPA (and other proteins such as RPA or XPC) might bind to the lesion coordinately with TFIIH to facilitate ATP-dependent opening around a damaged site. This opening would present XPG and ERCC1–XPF nucleases with suitable structures on each side. Consistent with this model, the opening detected by KMnO₄ footprinting did not require the incision activity of XPG itself.

The results presented here represent the first available information about changes to DNA structure surrounding a lesion during human NER. As well as revealing opening by increased sensitivity to oxidation or nuclease cleavage, the pattern observed in footprinting might reflect shielding or protection of certain residues due to protein binding. The particular pattern of opening might also depend on the sequence context and the type of lesion being repaired, since different types of damage cause their own particular distortions of the double helix. Detailed footprinting analysis of extracts from all the XP groups and the use of purified proteins should yield information on the stepwise mechanism of opening before nuclease cleavage and a view of the architecture of unwound intermediates during the repair reaction.

Materials and methods

Materials

S1 nuclease and calf intestinal alkaline phosphatase were obtained from Boehringer Mannheim, P1 nuclease and mung bean nuclease from Pharmacia and other DNA modification enzymes from New England Biolabs. MgCl₂ and MnCl₂ (ultrapure) were from Fluka and radionucleotides (>3000 Ci/mmol) from Amersham. HeLa cells and the lymphoblastoid cell lines GM2345 (XP-A) and XPG83 (XP-G) were cultured and extracts prepared as described (Wood *et al.*, 1995).

Proteins

Human RPA, PCNA and XPA were purified as described (Aboussekhra *et al.*, 1995). Human XPG protein was expressed from recombinant baculovirus (O'Donovan *et al.*, 1994b) and purified by phosphocellulose and hydroxyapatite column chromatography according to the described procedure, except that the buffer used for hydroxyapatite chromatography was 0.2 M KH₂PO₄, pH 6.8, 10% glycerol, 0.1% octylglucoside, 50 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin A and 0.1 mM phenylmethylsulfonylfluoride. Follow-

ing elution with a gradient of from 0.2 to 0.5 M phosphate, peak fractions were pooled and dialysed overnight at 4°C against 20 mM HEPES, pH 7.4, 0.2% Na azide, concentrated using a Centricon 30 apparatus (Amicon Inc.), adjusted to 25 mM HEPES, pH 7.5, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 0.02% NP-40, 50% glycerol and stored at −80°C. The polypeptide was ~95% pure as judged by Coomassie Blue staining. Purified XPG was active in DNA repair assays and could complement XPG-deficient extracts (data not shown). Polyclonal antiserum against a His-tagged 14 kDa N-terminal polypeptide of XPG protein (O'Donovan *et al.*, 1994a) was a gift from D.Scherly and S.Clarkson. A monoclonal antibody, 8H7, was raised against purified XPG and stored in 0.1 M Tris–HCl, pH 8.

Oligonucleotide substrates

Oligonucleotides were prepared by phosphoramidite chemistry using an Applied Biosystems model 380B DNA synthesizer and purified from denaturing polyacrylamide gels before use. The sequences are summarized in Table I. Splayed arm (pseudo-Y), duplex and bubble substrates, diagrammed in Figures 1, 2 and 4, were prepared essentially as described (Davies *et al.*, 1995) by annealing the oligonucleotide pairs listed below, one bearing a radioactive phosphate on the 5'-terminus:

Substrate: annealed strands

duplex: ds/ds comp	splayed arm: Y/Y comp	single-strand: Y
BS-A: BS1/BS2	BS-B: BS3/BS4	BS-C: BS5/BS6
BS-D: BS7/BS8	BS-E: BS9/BS10	BS-F: BS11/BS12
BS-G: BS13/BS14	BS-H: BS15/BS16	

Radiolabelled oligonucleotide (~200 ng) was mixed with a 4-fold molar excess of the indicated unlabelled partner in buffer containing 70 mM Tris–HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT. The DNA was heated to 95°C for 2 min, 68°C for 10 min, 37°C for 10 min and room temperature for 15 min, then purified on non-denaturing 10% polyacrylamide gels in TBE buffer (90 mM Tris–borate, pH 8.5, 2 mM EDTA) at 4°C. Substrate bands were electroeluted for 2 h at 4°C using a Schleicher & Schuell Biotrap apparatus in TBE buffer. Samples were dialysed overnight against TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) containing 50 mM NaCl and stored at −20°C. To prepare markers, Maxam–Gilbert sequencing reactions were performed on the radiolabelled strands.

Nuclease assays

For standard assays, the indicated amount of recombinant XPG protein was incubated for 90 min at 37°C in 20 µl containing ~16 fmol radiolabelled substrate, 25 mM HEPES, pH 6.8, 10% glycerol, 2 mM MgCl₂, 50 µg/ml bovine serum albumin (BSA) and 1 mM DTT. Concentrations of mono- or divalent cations were adjusted as indicated. Reactions were stopped by adding an equal volume of gel loading buffer (90% formamide, 1× TBE, 0.25% bromophenol blue). Samples were heated to 95°C for 3 min and the products separated on polyacrylamide gels containing 8.3 M urea: 12% gels were fixed in 5% methanol, 5% acetic acid for 15 min and dried prior to autoradiography or quantification using a PhosphorImager (Molecular Dynamics Inc.); in order to retain small oligonucleotides, higher percentage gels were processed directly without fixing (15 and 20%) and drying (20%).

Preparation of marker oligonucleotides

The 3'→5' exonuclease activity of bacteriophage T4 DNA polymerase (reacted under standard XPG assay conditions, no dNTPs present) was used to release radiolabelled mononucleotide, [α-³²P]dCMP, from a duplex DNA molecule bearing a single radiolabelled C residue incorporated at its 3'-terminus. The oligonucleotides d(OHCP₃COH), d(OHCP₃CP₃AOH), d(OHCP₃CP₃AGOH) and d(OHCP₃CP₃AGpTOH), were produced by chemical synthesis, radiolabelled separately on their 5'-termini using [γ-³²P]ATP and polynucleotide kinase, gel purified and placed in reaction buffer prior to loading on the gel.

Gel filtration analysis

Aliquots of 11 µg purified XPG protein were applied to a Superose 12 gel filtration column (SMART System; Pharmacia) equilibrated at 4°C with buffer A containing 25 mM HEPES, pH 7.4, 0.1 mM EDTA, 1 mM DTT, 20% glycerol and either 0.2 or 1 M KCl. SDS–PAGE and Coomassie Blue staining identified XPG-containing fractions. Fractions from the column run in 0.2 M salt were assayed directly by adding 1 µl (containing ~50 ng XPG in peak fractions) to a 20 µl reaction. Fractions from the column run in 1 M salt were first diluted 5-fold in buffer A without salt. BSA was added as a carrier and the volume of each fraction

Table I. Oligonucleotide sequences

Oligonucleotide	Sequence
61-mer ds	5'-GACGCTGCCGAATTCTACCAAGTGCCTTGCTAGGACATCTTTGCCACCTGCAGGTTACCC-3'
61-mer ds comp	5'-GGGTGAACCTGCAGGTGGGCAAAGATGTCCTAGCAAGGCACTGGTAGAATTCGGCAGCGTC-3'
61-mer Y	5'-TACACTGCCGAATTCTACCAAGTGCCTTGCTAGGACATCTTTGCCACCTGCAGGTTACCC-3'
62-mer Y comp	5'-TGGGTGAACCTGCAGGTGGGCAAAGATGTCCATCTGTTGTAATCGTCAAGCTTTATGCCGTT-3'
90-mer BS1	5'-CCAGTGATCACATACGCTGTGCTAGGACACCCCCCCCCCCCCCCCCCCCCCCCCCCCCAGCGCCACGTTGTCT-GCCACGTTGACCG-3'
90-mer BS2	5'-CGGTCAACGTGGGACAGACAACGTGGCGCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTCTTAGCACAGCGT-ATGTGATCACTGG-3'
90-mer BS3	5'-CCAGTGATCACATACCAAGCTGTGTGCTAGGACACCCCCCCCCCCCCCCCCCCCCCCCCAGCGCCACGTTGTCTGCGGAT-GCCACGTTGACCG-3'
90-mer BS4	5'-CGGTCAACGTGGTTCATCCGCGACAACGTGGCGCTGTTTTTTTTTTTTTTTTTTTTTTTGTGTCTTAGCACAGCAGCTGGT-ATGTGATCACTGG-3'
90-mer BS5	5'-CCAGTGATCACATACCAAGCTATGCTGTGCTAGGACACCCCCCCCCCCCCCCCCCCCCAGCGCCACGTTGTCTGCGGATA-ATGCCACGTTGACCG-3'
90-mer BS6	5'-CGGTCAACGTGGGCAATTATCCGCGACAACGTGGCGCTGTTTTTTTTTTTTTTTTTGTGTCTTAGCACAGCATAGCT-GGTATGTGATCACTGG-3'
90-mer BS7	5'-CCAGTGATCACATACCAAGCTATGACGCTGTGCTAGGACACCCCCCCCCCCCCAGCGCCACGTTGTCTGCGGATA-ACATGCCACGTTGACCG-3'
90-mer BS8	5'-CGGTCAACGTGGGCGATGTTATCCGCGACAACGTGGCGCTGTTTTTTTTTTTGTGTCTTAGCACAGCGTCATAGC-TGGTATGTGATCACTGG-3'
90-mer BS9	5'-CCAGTGATCACATACCAAGCTATGACGCTGTGCTAGGACACCCCCCCCCAGCGCCACGTTGTCTGCGGATAACAATGT-GCCACGTTGACCG-3'
90-mer BS10	5'-CGGTCAACGTGGGCGACATTGTTATCCGCGACAACGTGGCGCTGTTTTTTTTTTTGTGTCTTAGCACAGCTGGTCATAG-CTGGTATGTGATCACTGG-3'
85-mer BS11	5'-CCAGTGATCACATACGCTGTGCTAGGACACCCCCCCCCCCCCCCCCCCCCCCCCCCCCAGCGCCACGTTGTCT-GCCACGTT-3'
85-mer BS12	5'-AACGTGGGACAGACAACGTGGCGCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTCTTAGCACAGCGTA-TGTGATCACTGG-3'
80-mer BS13	5'-CCAGTGATCACATACGCTGTGCTAGGACACCCCCCCCCCCCCCCCCCCCCCCCCCCCCAGCGCCACG-TTGTCTGCCC-3'
80-mer BS14	5'-GGGACAGACAACGTGGCGCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTCTTAGCACAGCGTATG-TGATCACTGG-3'
75-mer BS15	5'-CCAGTGATCACATACGCTGTGCTAGGACACCCCCCCCCCCCCCCCCCCCCCCCCCCCCAGCGCCACGT-TGTC-3'
75-mer BS16	5'-GACAACGTGGCGCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTCTTAGCACAGCGTATGTGATCACTGG-3'

reduced 5-fold by centrifugation at 4°C using Microcon 30 filtration units (Amicon Inc.). Control experiments performed in parallel showed that this procedure did not affect the nuclease activities of purified XPG (data not shown).

DNA substrates containing a 1,3-intrastrand d(GpTpG)-cisplatin crosslink

Covalently closed circular DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink (Pt-GTG) or lacking damage (Con-GTG) was produced as described (Moggs *et al.*, 1996). For chemical footprinting of repair reactions, closed circular DNA was cleaved at the *Ava*II site 140 bp downstream of the platinum lesion and radiolabelled uniquely at the adjacent 3'-end using [α - 32 P]TTP and *E. coli* DNA polymerase I (Klenow fragment) or dephosphorylated and then radiolabelled on both 5'-ends using [γ - 32 P]ATP and T4 polynucleotide kinase. The linear DNA was purified from the labelling reactions by low speed centrifugation on a G-50 Sephadex (Pharmacia) spin column equilibrated in TE buffer.

Probing for single-stranded DNA in DNA repair reactions

Repair reactions (40 μ l) contained 200 ng Pt-GTG and 100–200 μ g of the indicated whole cell extract (Wood *et al.*, 1995) in repair buffer containing 45 mM HEPES-KOH, pH 7.8, 70 mM KCl, 7.5 mM MgCl₂, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μ M each dATP, dCTP, dGTP and TTP, 40 mM phosphocreatine (di-Tris salt), 2.5 μ g creatine phosphokinase, 3.4% glycerol and 18 μ g BSA. Some experiments (as indicated) used repair buffer without dNTPs and with or without ATP and the ATP regenerating system. Cell extract and any complementing proteins were added to the buffer on ice and preincubated at 30°C for 5 min. DNA substrate was added and incubation continued at 30°C. For the Southern hybridization incision assay, 200 ng unlabelled DNA was added to the reaction and analysis was performed as described (Sijbers *et al.*, 1996). KMnO₄ footprinting methods were adapted from published procedures (Borowiec *et al.*, 1988; Visse *et al.*, 1994; Jiang and Gralla, 1995). 3'- (damaged strand) or 5'- (non-damaged strand) end-labelled DNA (~200 ng) was added to the repair reaction following the preincuba-

tion. At the indicated times, some reactions were made 6 mM in KMnO₄ (Sigma) by adding 2 μ l of a 120 mM stock. After 1 min at 30°C, oxidation was quenched by the addition of 2-mercaptoethanol to 1 M. Reactions were terminated by the addition of 24 mM EDTA, 0.5% SDS and 50 μ g/ml yeast tRNA and the DNA was purified by proteinase K digestion, organic extraction and ethanol precipitation. To cleave at oxidized residues, the purified DNA was resuspended in 70 μ l 1 M piperidine and treated at 90°C for 25 min. The piperidine was removed by evaporation with dH₂O and the pellet resuspended in loading buffer, separated on a 6% polyacrylamide sequencing gel, fixed, dried and exposed to Kodak X-Omat film or a PhosphorImager. As DNA size markers, dideoxy sequencing ladders of M13mp18 DNA were prepared using a 5'-radiolabelled -40 sequencing primer and the Sequenase v 2.0 kit (US Biochemicals) according to the manufacturer's instructions. P1 nuclease digestion was carried out as described (Kowalski, 1984; Bramhill and Kornberg, 1988).

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