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Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase β and the XRCC1 protein

Yoshiko Kubota[1], Rachel A.Nash, Arne Klungland, Primo Schär, Deborah E.Barnes and Tomas Lindahl[2]

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK

[1]Present address: Department of Neurochemistry and Molecular Biology, Institute of Development, Aging and Cancer, Tohoku University, Sendai 980-77, Japan

[2]Corresponding author

Repair of a uracil–guanine base pair in DNA has been reconstituted with the recombinant human proteins uracil-DNA glycosylase, apurinic/apyrimidinic endonuclease, DNA polymerase β and DNA ligase III. The XRCC1 protein, which is known to bind DNA ligase III, is not absolutely required for the reaction but suppresses strand displacement by DNA polymerase β, allowing for more efficient ligation after filling of a single nucleotide patch. We show that XRCC1 interacts directly with DNA polymerase β using far Western blotting, affinity precipitation and yeast two-hybrid analyses. In addition, a complex formed between DNA polymerase β and a double-stranded oligonucleotide containing an incised abasic site was supershifted by XRCC1 in a gel retardation assay. The region of interaction with DNA polymerase β is located within residues 84–183 in the N-terminal half of the XRCC1 protein, whereas the C-terminal region of XRCC1 is involved in binding DNA ligase III. These data indicate that XRCC1, which has no known catalytic activity, might serve as a scaffold protein during base excision-repair. DNA strand displacement and excessive gap filling during DNA repair were observed in cell-free extracts of an XRCC1-deficient mutant cell line, in agreement with the results from the reconstituted system.

Keywords: deamination of DNA/DNA polymerase β/ DNA repair

Introduction

Endogenous DNA base damage generated by hydrolysis or oxidation is a major threat to the genetic integrity of cells. The main pathway of repair of such damage involves several distinct DNA glycosylases which can excise a variety of altered bases in free form by hydrolytic cleavage of base–sugar bonds, an apurinic/apyrimidinic (AP) endonuclease for incision at abasic sites, an excision function to remove base-free sugar phosphate residues, a DNA polymerase for short patch gap filling and a DNA ligase (Lindahl, 1976). The entire reaction involves the replacement of a single damaged nucleotide residue and has been reproduced *in vitro* with cell-free extracts from *Escherichia coli* and human cells (Wiebauer and Jiricny 1990; Dianov *et al.*, 1992; Singhal *et al.*, 1995). Reconstitution of the reaction with purified enzymes from *E.coli* has also been achieved (Dianov and Lindahl, 1994). Here, we extend this approach to reconstitution of the base excision-repair (BER) pathway with purified recombinant proteins of human origin, employing DNA with a defined uracil residue as a substrate. Uracil residues in DNA can arise by spontaneous deamination of cytosine, and these potentially mutagenic lesions are repaired efficiently *in vivo* by a BER process initiated by uracil-DNA glycosylase.

In variants of the BER pathway, some DNA glycosylases active on oxidized nucleotides possess intrinsic AP lyase activity for incision at the 3′ side of the base-free site. This allows for the generation of a single nucleotide gap by AP endonuclease cleavage at the 5′ side of the lesion (Demple and Harrison, 1994). Furthermore, longer DNA repair patches can be generated in a minor alternative gap filling procedure which may involve a proliferating cell nuclear antigen (PCNA)-dependent DNA polymerase in higher eukaryotes (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996).

The general strategy of the main BER pathway and the initial reaction steps have been highly conserved from microorganisms to man. However, the later events during BER are different in higher eukaryotes. The 39 kDa mammalian DNA polymerase β, which is the enzyme responsible for gap filling during BER (Sobol *et al.*, 1996), has an 8 kDa basic N-terminal domain which can excise a 5′-terminal base-free deoxyribose phosphate (dRp) residue from incised abasic sites by a β-elimination mechanism (Matsumoto and Kim, 1995; Piersen *et al.*, 1996). In contrast, *E.coli* requires a separate enzyme for excision of dRp in free form (Dianov *et al.*, 1994), and *Saccharomyces cerevisiae* differs from both mammalian cells and *E.coli* in generating longer DNA repair patches during BER (Wang *et al.*, 1993; Blank *et al.*, 1994). Genetic evidence implicates the XRCC1 protein as having an important role in mammalian BER (Thompson *et al.*, 1990). XRCC1 has no known catalytic function but forms a tight complex with one of the nuclear ligases, DNA ligase III (Caldecott *et al.*, 1994, 1995). No counterparts to either XRCC1 or DNA ligase III have been detected in *S.cerevisiae* by biochemical experiments or in screening the recently completed sequence of the yeast genome. Reconstitution of the repair reaction with purified human proteins and analysis of an XRCC1-deficient mutant cell line provide insights into BER in mammalian cells.

Results

Reconstitution of DNA base excision-repair with purified human proteins

We have reconstituted BER *in vitro* with the purified human proteins, uracil-DNA glycosylase (UDG), AP

endonuclease (HAP1), DNA polymerase β and DNA ligase III. The biochemical role of the XRCC1 protein has also been investigated. The recombinant human proteins used are shown in Figure 1. Uracil residues in DNA are repaired efficiently *in vivo* by BER, and double-stranded oligonucleotides containing a centrally placed U·G base pair have been employed as substrates for *in vitro* reactions (Dianov *et al.*, 1992; Dianov and Lindahl, 1994; Singhal *et al.*, 1995). Here, a double-stranded oligonucleotide substrate 5′-^{32}P-labelled on the 41 nucleotide (nt) uracil-containing strand was used, and visualized following denaturing polyacrylamide gel electrophoresis and autoradiography (Figure 2).

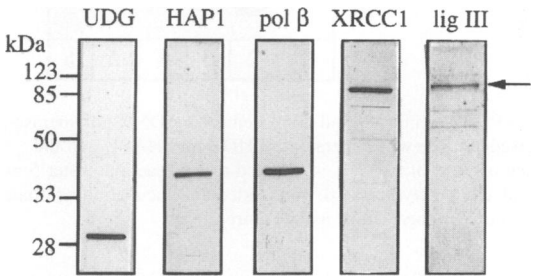

Fig. 1. Human proteins used in reconstitution of base excision-repair. Proteins were purified as described in Materials and methods, electrophoresed in 12% SDS–polyacrylamide gels and visualized by silver staining. Molecular weight markers (kDa) are shown. The arrow indicates the full-length protein where lower molecular weight forms are present. pol β, DNA polymerase β; lig III, DNA ligase III.

An AP site generated by the action of UDG was incised at the 5′ side by the hydrolytic action of HAP1 to generate a labelled 20 nt oligonucleotide (Figure 2A, lane 6; Figure 2B). A double-stranded oligonucleotide containing a C·G base pair in place of the U·G pair was refractory to the action of UDG and HAP1 (Figure 2A, lanes 1 and 2). No strand interruption in the uracil-containing substrate was introduced by either UDG or HAP1 alone (Figure 2A, lanes 4 and 5). After generation of an incised AP site, DNA polymerase β was able to fill in a single nucleotide, replacing the U with a C and generating a labelled 21 nt oligonucleotide as the main product (Figure 2A, lane 7; Figure 2B). This 21mer could be ligated completely to generate the 41 nt final product (Figure 2A, lane 8), confirming that DNA polymerase β is able to remove a 5′ dRp residue from an incised AP site (Matsumoto and Kim, 1995). Kinetic experiments showed that joining was achieved immediately after gap filling in the presence of excess DNA ligase, without accumulation of the 21 nt reaction intermediate, indicating that dRp removal by DNA polymerase β preceded or was more efficient than the polymerization step (data not shown).

Although DNA polymerase β primarily filled in a single nucleotide patch, it was also able to generate patches two or three nucleotides in length, leading to labelled oligonucleotides of 22 and 23 nt (Figure 2A, lane 7). These could not be ligated (Figure 2A, lane 8), and therefore apparently reflected displacement of the cleaved strand by the polymerase to generate an overhang structure.

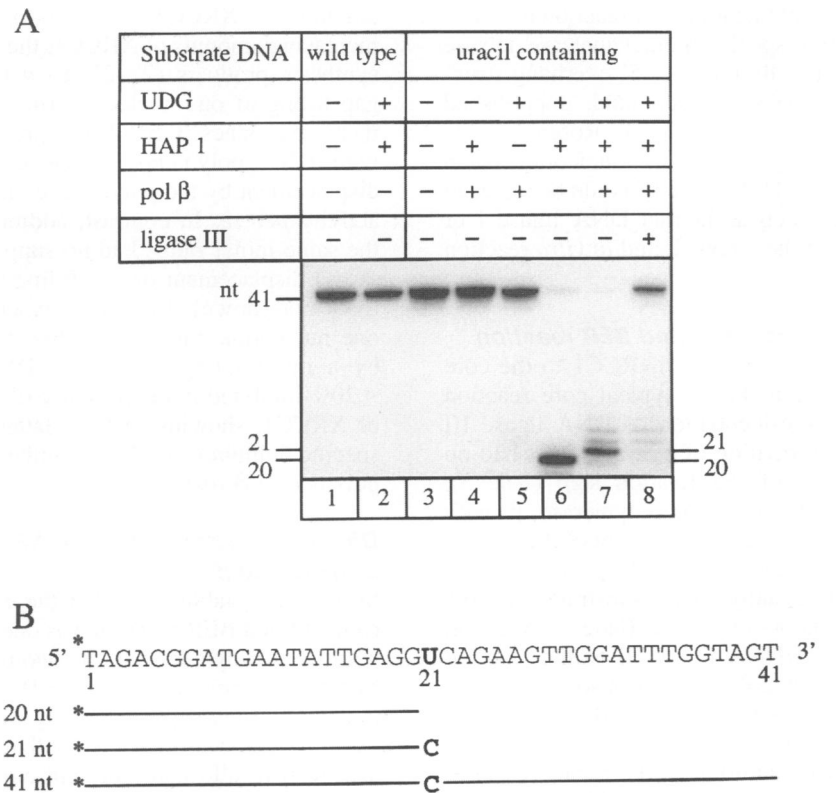

Fig. 2. Reconstitution of DNA base excision-repair with human proteins. (**A**) BER assay. Substrate DNA was incubated with the human enzymes indicated and reaction products analysed by autoradiography after separation in 20% denaturing polyacrylamide gels. Reaction conditions were as described in Materials and methods. The sizes (nt) and positions of reaction products are indicated. (**B**) Substrate DNA and predicted partial or complete reaction products. The sequence of the uracil-containing strand is given and the uracil residue is shown in bold typeface; the wild-type substrate and the repaired uracil-containing substrate have a C in place of the U at this position. Asterisks indicate the position of the ^{32}P label; nucleotides are numbered from the 5′ end.

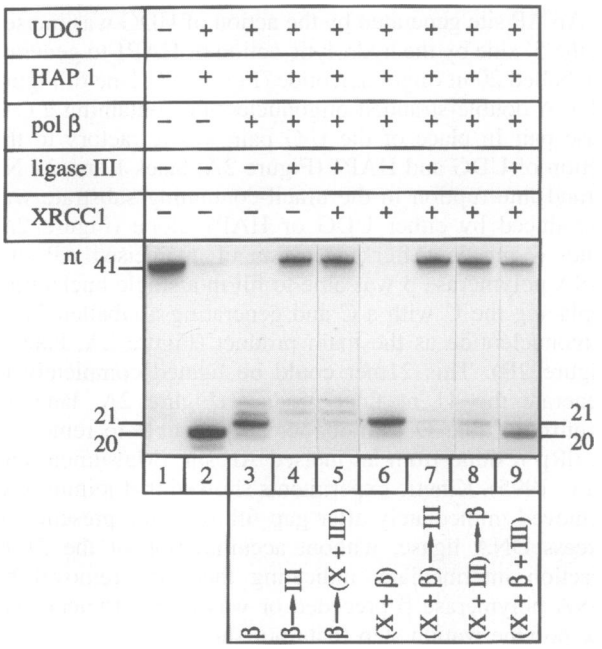

Fig. 3. Reconstituted core BER reaction with addition of XRCC1 protein. After generation of an incised AP site by UDG and HAP1, enzymes were added as indicated. Parentheses indicate pre-incubation of proteins at 37°C for 5 min before addition to the reaction mixture; arrows indicate the order of additions. β, DNA polymerase β; X, XRCC1; III, DNA ligase III. All other details as in Figure 2.

In agreement with this notion, the longer repair patches could be ligated after supplementation of reaction mixtures with the purified structure-specific human nuclease, DNase IV/FEN-1, which specifically removes 5′ overhang structures at DNA strand breaks (A.Klungland, unpublished data; see Harrington and Lieber, 1994; Robins *et al.*, 1994). In the final joining of the one nucleotide patch (Figure 2, lane 8), human DNA ligase III could be replaced by other DNA ligases such as human DNA ligase I or phage T4 DNA ligase in the reconstituted *in vitro* reaction (data not shown).

Role of XRCC1 in the reconstituted BER reaction

We investigated the effect of adding XRCC1 to the core reconstituted BER reaction. In the typical core reaction (Figure 3, lanes 1–4), pre-incubation of DNA ligase III with XRCC1 to allow formation of a heterodimer had no effect on the efficiency of the ligation step (lane 5). However, pre-incubation of DNA polymerase β with XRCC1 greatly reduced strand displacement of the incised double-stranded oligonucleotide during the polymerization step (lane 6) such that virtually all the substrate was able to be ligated into the final 41 nt product (lane 7). Addition of pre-incubated XRCC1/DNA ligase III to the reaction mixture followed by DNA polymerase β also allowed for efficient repair with little or no DNA strand displacement (lane 8). When all three recombinant proteins were pre-incubated together before addition to the repair reaction, the DNA gap filling step was partly inhibited, possibly by interference of remaining monomeric DNA ligase III at the single nucleotide gap (lane 9). Since the XRCC1 protein and DNA ligase III form a heterodimer in cells, the data shown in lane 8 seem best representative of the situation *in vivo*.

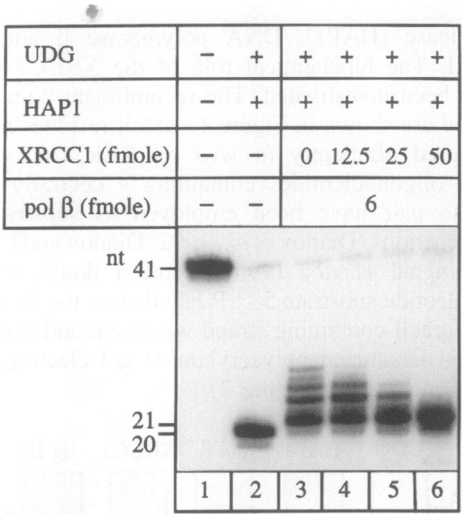

Fig. 4. XRCC1 inhibits strand displacement by DNA polymerase β. An incised AP site was generated by UDG and HAP1, and the indicated amount of XRCC1 was added to the reaction. After 5 min, 6 fmol of DNA polymerase β were added and incubation continued for 10 min. All other details as in Figure 2.

To analyse further the effect of XRCC1 on the gap filling reaction catalysed by DNA polymerase β, an abridged version of the BER reaction without the ligation step was set up; after generation of the incised AP site with UDG and HAP1 (Figure 4, lanes 1 and 2), DNA polymerase β (twice the amount used in the standard reaction) was added after pre-incubation with various amounts of XRCC1 (lanes 3–6). With the addition of increasing amounts of XRCC1, the proportion of extended synthesis products (22–25 nt) was suppressed, whereas gap filling of one nucleotide (to form the 21 nt species) increased (lanes 3–6). Thus, pre-incubation of XRCC1 with DNA polymerase β specifically prevented strand displacement by the polymerase but not DNA polymerase activity *per se*. In contrast, addition of histone H1 over the same molar range had no suppressive effect on either strand displacement or gap filling by DNA polymerase β (data not shown). In kinetic experiments, filling in of a one nucleotide gap in a double-stranded oligonucleotide by a rate-limiting amount of DNA polymerase β was <20% inhibited in the presence of a 10-fold molar excess of XRCC1, showing that the latter protein is not a non-specific inhibitor of DNA synthesis catalysed by DNA polymerase β (data not shown).

Direct interaction between XRCC1 and DNA polymerase β

In order to establish whether the effect of XRCC1 in the reconstituted BER reaction was due to physical interaction with DNA polymerase β, the two proteins were examined by affinity precipitation and far Western blotting analyses. In the affinity precipitation assay, histidine-tagged XRCC1 (XRCC1-His) was incubated with recombinant DNA polymerase β to allow any protein complexes to form, prior to affinity precipitation with nickel–agarose beads. After extensive washing to remove non-specifically associated proteins, XRCC1-His and bound proteins were eluted with 250 mM imidazole, and analysed by SDS–PAGE and silver staining. Bovine serum albumin (BSA) and NaCl (100 mM) were included in the reaction; the BSA was

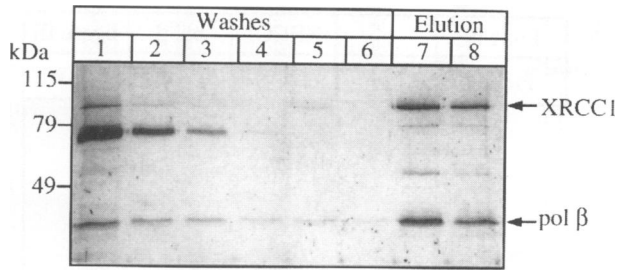

Fig. 5. Affinity precipitation of DNA polymerase β with histidine-tagged XRCC1. XRCC1-His was incubated with DNA polymerase β and affinity precipitated with Ni-NTA–agarose. Agarose beads were washed six times with 20 mM imidazole (lanes 1–6) and bound proteins eluted with two successive 250 mM imidazole elutions (lanes 7 and 8). Aliquots from washes and elutions were analysed by SDS–PAGE and silver staining. Molecular mass markers (kDa) are shown. The ~70 kDa band in wash fractions is BSA, which was added to reactions.

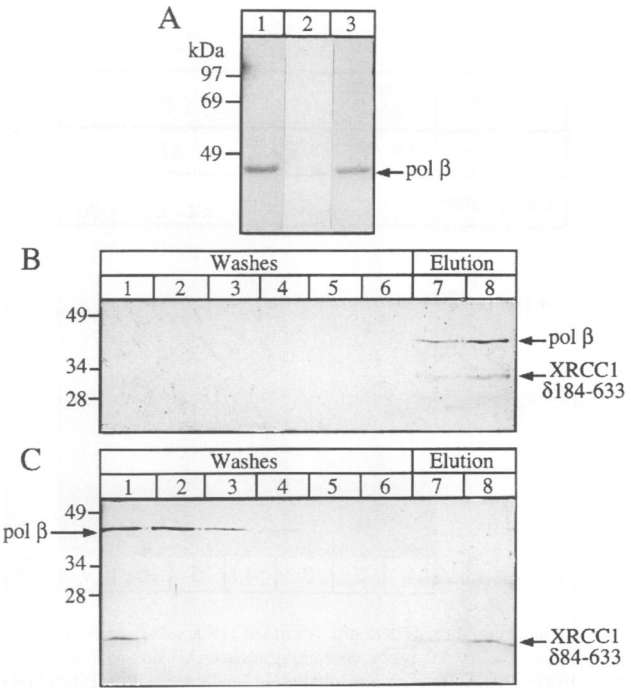

Fig. 6. Interaction of full-length and mutant XRCC1 proteins with DNA polymerase β. (**A**) Far Western analysis. DNA polymerase β was electrophoresed in a 10% denaturing polyacrylamide gel, transferred to a nitrocellulose filter and hybridized with [^{32}P]XRCC1 probes, as follows. Lane 1: full-length XRCC1; lane 2: XRCC1 δ56–374; lane 3: XRCC1 δ468–633. (**B** and **C**) Affinity precipitation analysis. DNA polymerase β was affinity precipitated with histidine-tagged XRCC1 δ184–633 (B) or XRCC1 δ84–633 (C). Experimental details as in Figure 5, except that the BSA band is not seen in the portion of the gel shown.

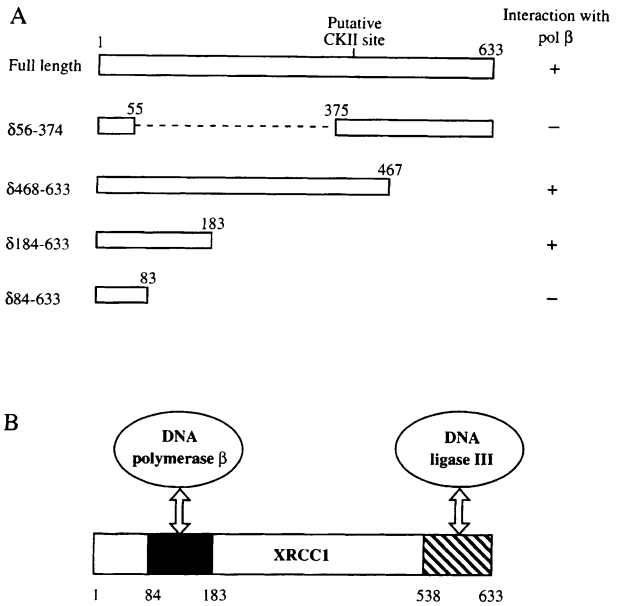

Fig. 7. XRCC1 constructs and interactions. (**A**) Deletion mutants. Open boxes represent expressed regions and dashed lines indicate deleted sequences, with amino acids numbered. The position of a CKII consensus site, allowing generation of [^{32}P]XRCC1 probes for far Western analyses, is indicated. Interaction with DNA polymerase β is summarized from the data in Figures 5 and 6. (**B**) Schematic diagram of the XRCC1 protein, showing the regions of interaction with DNA polymerase β (within amino acids 84–183) and DNA ligase III (538–633). The binding site for DNA ligase III was determined previously (R.A.Nash, K.W.Caldecott, D.E.Barnes and T.Lindahl, submitted).

eluted in the wash fractions (Figure 5, lanes 1–3). XRCC1 was able to bind DNA polymerase β in the affinity precipitation assay, as indicated by co-elution of the two proteins (Figure 5, lanes 7 and 8). XRCC1 also bound DNA ligase III under similar conditions, as reported previously (Caldecott *et al.*, 1995; Wei *et al.*, 1995).

The interaction between XRCC1 and DNA polymerase β was examined further using XRCC1 proteins with internal or C-terminal deletions (Figures 6 and 7). A full-length ^{32}P-labelled XRCC1 probe was able to detect DNA polymerase β on far Western blots (Figure 6A, lane 1). These data confirm the affinity precipitation results. A truncated XRCC1 protein with a large deletion in the N-terminal region (δ56–374; Figure 7A) was unable to bind DNA polymerase β in the far Western assay (Figure 6A, lane 2), while a truncated form of XRCC1 lacking the C-terminal 166 amino acids (δ468–633; Figure 7A) was able to bind as efficiently as full-length XRCC1 (Figure 6A, lane 3). This indicates that DNA polymerase β binds to the N-terminal region of XRCC1, within amino acids 56–374.

To delineate further the region of interaction with DNA polymerase β, two severely truncated forms of XRCC1 were analysed, δ184–633 and δ84–633, representing the N-terminal 183 or 83 amino acids of the protein, respectively (Figure 7A). As these truncated proteins lack the casein kinase II (CKII) consensus sequence and could not be ^{32}P-labelled as probes for far Western analysis (Figure 7A), the interaction between DNA polymerase β and δ184–633 or δ84–633 was analysed by the affinity precipitation assay. DNA polymerase β bound to δ184–633 and co-eluted with the truncated XRCC1-His protein (Figure 6B, lanes 7 and 8). However, DNA polymerase β did not bind to the shorter δ84–633 protein, did not co-elute with it (Figure 6C, lanes 7 and 8) but was detected in the wash fractions (Figure 6C, lanes 1–4). This indicates that the N-terminal 183 amino acids of XRCC1 are sufficient for binding to DNA polymerase β and that the site of interaction is located between amino acids 84 and 183 in the N-terminal region of XRCC1 (Figure 7B). DNA ligase III binds to the C-terminal 96 amino acids of XRCC1 (R.A.Nash, K.W.Caldecott, D.E.Barnes and T.Lindahl, submitted; Figure 7B). In contrast to the interaction

between DNA polymerase β and XRCC1, DNA polymerase β did not bind detectably to DNA ligase III in the affinity precipitation assay (data not shown).

A physical interaction between DNA polymerase β and XRCC1 *in vivo* was demonstrated in yeast using the two-hybrid system (Chien *et al.*, 1991). Full-length XRCC1 and DNA polymerase β proteins were fused to the DNA binding domain (DNA-BD) and the activation domain (AD) of the GAL4 transcriptional activator, respectively. Different pair-wise combinations of DNA-BD and AD constructs were tested for their ability to induce β-galactosidase activity after co-transformation of the *S.cerevisiae* host strain. Of all combinations tested, only the DNA-BD-XRCC1/AD-β-polymerase pair was able to induce β-galactosidase activity; combinations of the DNA-BD vector with the AD vector, DNA-BD-XRCC1 with the AD vector, the DNA-BD vector with AD-β-polymerase and DNA-BD-human lamin C with AD-β-polymerase (as a negative control) were not able to induce activity. The relative amounts of β-galactosidase induced in DNA-BD-XRCC1/AD-β-polymerase-expressing cells and the positive control (DNA-BD–murine p53/AD–SV40 large T antigen-expressing cells) were similar as judged from the kinetics and the intensity of blue colour development by β-galactosidase-catalysed conversion of X-gal (data not shown). These results indicate a specific and stable physical interaction of XRCC1 with DNA polymerase β *in vivo*.

Gel retardation assays

Having established protein–protein interactions between XRCC1 and both DNA polymerase β and DNA ligase III, we next investigated how these three proteins might interact independently or in concert with their substrate in the reconstituted BER reaction. The ^{32}P-labelled double-stranded uracil-containing oligonucleotide substrate used in the BER reaction was recovered after generation of incised AP sites with UDG and HAP1 was complete; this material was used as a probe in gel retardation assays, and the double-stranded oligonucleotide with a C·G in place of the U·G base pair was used as a control (Figure 8A, lanes 1 and 2). Neither XRCC1 nor DNA ligase III bound detectably to either probe (Figure 8A, lanes 3, 4, 7 and 8). DNA polymerase β did not bind the control probe (Figure 8A, lane 6) but specifically bound at incised AP sites, forming a retarded complex with the ^{32}P-labelled probe (Figure 8A, lane 5). Furthermore, when DNA polymerase β was pre-incubated with XRCC1 before incubation with probe DNAs, the same probe was again specifically retarded by DNA polymerase β and, in addition, a supershifted band was observed (Figure 8B, lane 4). The ^{32}P label in the supershifted band was decreased by addition of unlabelled competitor (Figure 8B, lanes 5–7). These data indicate the formation of a complex of DNA polymerase β and XRCC1 at an incised AP site, confirming that DNA polymerase β and XRCC1 are able to interact, and show that DNA polymerase β is able to bind specifically to an incised AP site either on its own or when bound to XRCC1. Consistent with there being no interaction between DNA polymerase β and DNA ligase III, pre-incubation of DNA polymerase β with DNA ligase III did not lead to formation of a supershifted complex (Figure 8B, lane 1).

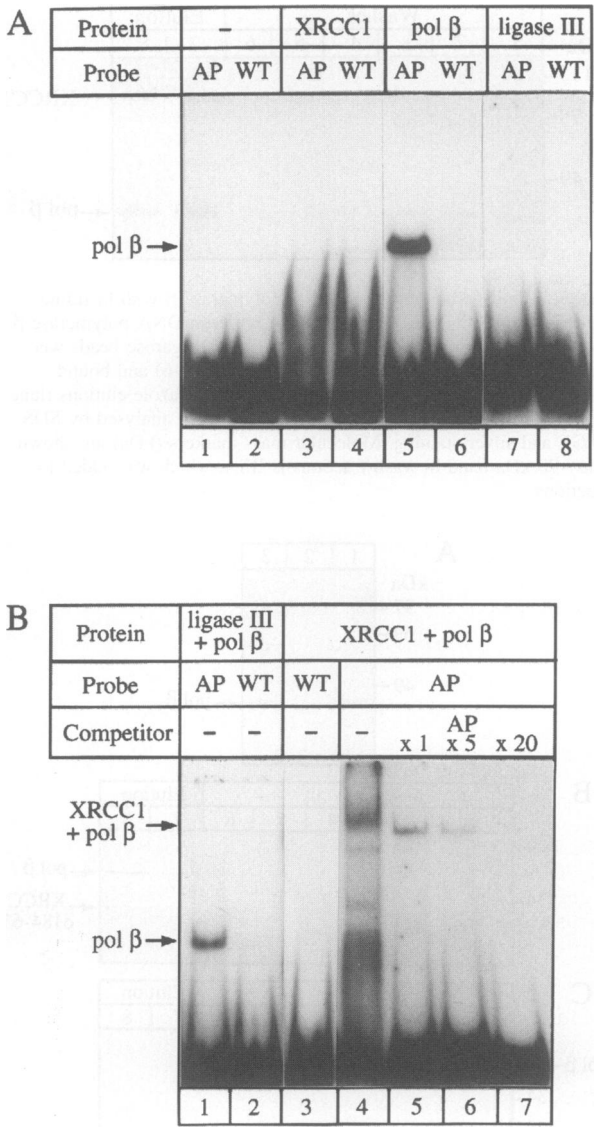

Fig. 8. Gel retardation of a double-stranded DNA substrate containing an incised AP site. AP, probe containing nicked AP site; WT, wild-type probe. (**A**) Proteins as indicated were incubated with ^{32}P-labelled AP or WT probes. (**B**) Combinations of proteins were pre-incubated at 37°C for 5 min before addition of probe DNA. The amount of non-radiolabelled competitor DNA was in 1, 5 or 20 times molar excess over the probe DNA, as indicated. The specific retarded complex formed between the AP probe and DNA polymerase β, and the supershifted complex formed with XRCC1, are indicated by arrows.

BER in extracts from XRCC1-deficient cells

Efficient repair of a uracil-containing double-stranded oligonucleotide substrate can be achieved with mammalian cell-free extracts (Figure 9). The reaction largely reflects the replacement of a single nucleotide residue (Dianov *et al.*, 1992; Singhal *et al.*, 1995). As with the reconstituted system, double-stranded oligonucleotide substrates with a central U·G or C·G base pair were employed; however, in this instance, oligonucleotide substrates were not 5′ ^{32}P-labelled, and the BER reaction was monitored through replacement of the U using [α-^{32}P]dCTP during the repair reaction (Figure 9B). In agreement with previous studies on human cell-free extracts (Satoh *et al.*, 1993), the BER reaction was dependent on the presence of a U·G base pair in the substrate (data not shown) and was promoted

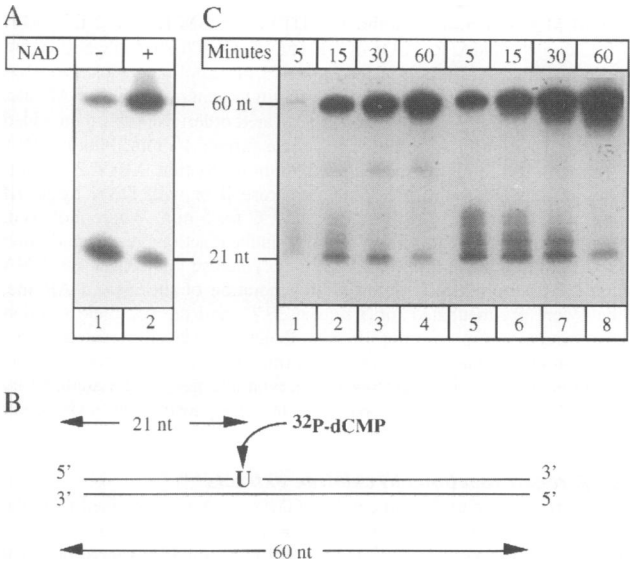

Fig. 9. BER in cell-free extracts and analysis of an XRCC1-deficient mutant cell line. (**A**) BER assay. Substrate DNA was incubated with a cell-free extract of the normal human lymphoblastoid cell line GM06315A for 30 min, in the absence (lane 1) or presence (lane 2) of 1 mM NAD, and the reaction products analysed by autoradiography after separation in a 20% denaturing polyacrylamide gel. The sizes and positions of reaction products are indicated. (**B**) Substrate DNA. Predicted reaction products resulting from replacement of the dUMP with a [^{32}P]dCMP residue are shown. (**C**) Analysis of a mutant CHO cell line. Substrate DNA was incubated with cell-free extracts in the presence of 1 mM NAD and the reaction products analysed by autoradiography after separation in a 20% denaturing polyacrylamide gel. The sizes and positions of reaction products are indicated. Incubations were for 5 min (lanes 1 and 5), 15 min (lanes 2 and 6), 30 min (lanes 3 and 7) or 60 min (lanes 4 and 8). Lanes 1–4, AA8 (wild-type); lanes 5–8, EM9 (XRCC1-deficient).

by the addition of NAD (Figure 9A). NAD is required for release of the abundant poly(ADP-ribose) polymerase from DNA strand interruptions (Zahradka and Ebisuzaki, 1982). In the absence of NAD, gap filling could still occur, but the final joining step was inhibited (Figure 9A).

Having established an effect of XRCC1 on the reconstituted reaction, we examined BER in cell-free extracts of the *xrcc1* mutant CHO cell line, EM9, in comparison with the AA8 wild-type parental line. EM9 cells contain no detectable XRCC1 protein (>10-fold decreased level) and have an ~4-fold decreased level of DNA ligase III (Caldecott *et al.*, 1995). The BER reaction could proceed to completion in extracts of both AA8 and EM9 (Figure 9C). The data indicate that the reduced activity of DNA ligase III in EM9 cells is either sufficient for the ligation step, or the enzyme is substituted by other DNA ligases in the extract. In the absence of XRCC1 protein, there was marked strand displacement activity in the EM9 extract (Figure 9C, lane 5), in agreement with data from the reconstituted system. In contrast to the reconstituted system, all synthesis products resulting from strand displacement ultimately could be ligated by the extract, and the accompanying increased level of incorporation of ^{32}P-labelled material into religated product in the EM9 extract showed that many patches longer than one nucleotide were generated (Figure 9C). These data indicate the presence in the cell extract of the structure-specific endonuclease, DNase IV/FEN-1 (Harrington and Lieber, 1994; Robins *et al.*, 1994), which would remove 5′ overhang structures at strand interruptions after displacement synthesis. The main result here was that strand displacement and excessive gap filling were seen in the absence of the XRCC1 protein.

Discussion

The major form of BER of uracil-containing DNA in human cells involves replacement of a single nucleotide residue. The reaction has been investigated in cell extracts (Dianov *et al.*, 1992; Singhal *et al.*, 1995) and has been reproduced here with purified human proteins. The enzymes employed in the present study are likely to catalyse the relevant events in human cells *in vivo*. Thus, the UDG protein accounts for >98% of the UDG activity in human cell extracts (Slupphaug *et al.*, 1995), although a minor contribution of the separate G·T- and G·U-specific DNA glycosylase as a back-up enzyme seems likely (Neddermann *et al.*, 1996). Similarly, the HAP1/APE endonuclease accounts for ~99% of incisions at AP sites in DNA in human cell extracts (Demple and Harrison, 1994). Evidence from rodent cells lacking DNA polymerase β defines the main role of this enzyme *in vivo* as participation in the BER pathway (Sobol *et al.*, 1996). Several DNA ligases have been detected in human cell nuclei (Wei *et al.*, 1995), and it is not yet firmly established which one is primarily active in BER. However, the occurrence of DNA ligase III in a heterodimer with the XRCC1 protein, and the genetic evidence for a role of XRCC1 in the BER pathway (Thompson *et al.*, 1990), make DNA ligase III a likely candidate. In the present cell-free BER system, different DNA ligases can substitute for each other, and such back-up reactions may also occur *in vivo*. A similar situation has been described for the gap filling step during nucleotide excision-repair *in vitro*; DNA polymerase ε seems best suited to catalyse the reaction, but DNA polymerase δ may also be employed (Shivji *et al.*, 1995).

By several experimental approaches, XRCC1 was found to interact specifically with DNA polymerase β, in addition to the previously known interaction with DNA ligase III. These data implicate XRCC1, which has no known catalytic function, as a scaffold protein recruiting and positioning other components during BER. In this role, XRCC1 may suppress unnecessary DNA strand displacement and potentially error-prone long patch gap filling, and so promote concerted repair events without accumulation of reaction intermediates. The XRCC1 protein may also interact specifically with other BER enzymes, so that a repair complex forms at AP sites. It seems unlikely, however, that a DNA glycosylase would be part of such a putative protein complex, because many different DNA glycosylases exist that recognize distinct base lesions, and AP sites are also introduced at a significant rate by non-enzymatic depurination under *in vivo* conditions. It would be difficult to attempt to isolate a DNA repair complex directly from cell nuclei without initial protein cross-linking, because the enzymes involved in BER require extraction with >0.2 M NaCl for solubilization, and the only salt-resistant protein–protein interaction remaining under such conditions is the complex between XRCC1 and DNA ligase III (Caldecott *et al.*, 1995). However, Prasad *et al.* (1996) have reported on a BER complex

from bovine testis regenerated after high salt extraction, which apparently includes UDG, the HAP1 endonuclease, DNA polymerase β and DNA ligase I. Binding of DNA ligase I to DNA polymerase β was observed, although the sites of interaction were not defined. The present data do not exclude a role for DNA ligase I in BER. However, DNA ligase I is largely sequestered in 'replication factories' within cell nuclei together with DNA polymerase α and other replication factors (Montecucco *et al.*, 1995), whereas enzymes such as HAP1, DNA polymerase β and DNA ligase III appear to be evenly distributed in cell nuclei.

The core BER reaction reconstituted here may be modulated *in vivo* by accessory factors, although there is no experimental evidence for the participation in this short patch repair reaction of DNA helicases, or replication factors such as RP-A and PCNA which are required in the more complex nucleotide excision-repair pathway (Aboussekhra *et al.*, 1995). Also to be considered are possible interactions of the BER machinery with poly-(ADP-ribose) polymerase, which can interfere with BER by binding to DNA strand interruptions (Satoh *et al.*, 1993). Several of the key proteins employed in the present study are essential for viability as judged by the embryonic-lethal phenotype of mice defective in HAP1 (Xanthoudakis *et al.*, 1996), DNA polymerase β (Sobol *et al.*, 1996) and XRCC1 (Tebbs *et al.*, 1996). Continuous repair of endogenous DNA damage by the BER pathway described here may be required to avoid unacceptable levels of genomic instability.

Materials and methods

Recombinant human enzymes

Recombinant human UDG derived from plasmid pTUNGΔ84 (Slupphaug *et al.*, 1995) was provided by H.Krokan (University of Trondheim, Norway). Recombinant human HAP1 with an N-terminal histidine tag was provided by I.Hickson (ICRF Unit, Institute of Molecular Medicine, Oxford, UK). Human DNA polymerase β was expressed from plasmid pWL-11 (obtained from S.Wilson, University of Texas, Galveston, TX) and purified as described (Abbotts *et al.*, 1988). Human XRCC1 with an N-terminal histidine tag was expressed from plasmid pET16BXH and purified as described (Caldecott *et al.*, 1995). Histidine-tagged recombinant human DNA ligase III was produced as described (R.A. Nash, K.W.Caldecott, D.E.Barnes and T.Lindahl, submitted). All proteins employed had been overexpressed in soluble and active form in *E.coli*.

Oligonucleotide substrates

For the reconstituted BER assay with purified proteins, single-stranded 41mer oligonucleotides, with a U or C residue at position 21, were prepared on a commercial DNA synthesizer, purified in 20% denaturing polyacrylamide gels, 5′-^{32}P-labelled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham), and each annealed to a complementary strand with a G residue opposite the U or C (Dianov *et al.*, 1992). For gel retardation assays, annealed double-stranded oligonucleotides were purified in 15% native polyacrylamide gels and treated with UDG and HAP1 to generate a nicked AP site (see below); a small aliquot was analysed in a 20% denaturing polyacrylamide gel to check for completion of the reaction, and the remainder was recovered by ethanol precipitation following phenol/chloroform extraction. For assays with cell-free extracts, 60mer oligonucleotides were prepared with a U or C residue at position 21, purified and annealed to a complementary strand as above, but were not 5′-^{32}P-labelled. Oligonucleotides were made with phosphorothioate bonds at terminal residues to suppress degradation by exonucleases.

Reconstituted DNA repair

The core repair reaction was carried out in three stages. The reaction mixture (5 μl) containing 40 mM HEPES–KOH (pH 7.8), 70 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 2 mM ATP, 20 μM each of dATP, dTTP, dGTP and dCTP (Pharmacia), 500 μg/ml DNase-free BSA, UDG (185 fmol), HAP1 (40 fmol) and substrate DNA (30 fmol) was incubated at 37°C for 5 min to generate a nicked AP site. DNA polymerase β (3 fmol, unless indicated otherwise) was then added and the reaction mixture incubated for a further 10 min. Finally DNA ligase III (160 fmol) was added for a 5 min incubation. XRCC1 (6 fmol) was added together with DNA polymerase β or with DNA ligase III after pre-incubation of the proteins at 37°C for 5 min. Where indicated, incubation with XRCC1–DNA ligase III in the reaction preceded addition of the DNA polymerase. When DNA polymerase β, XRCC1 and DNA ligase III were added together after generation of the incised AP site, they were pre-incubated for 5 min at 37°C, and the complete reaction mix incubated for a further 10 min. Reactions were terminated by the addition of an equal volume of 95% formamide/dyes. The products were fractionated in 20% denaturing polyacrylamide gels, and visualized by autoradiography or analysed on a PhosphorImager (Molecular Dynamics).

DNA repair reactions in cell-free extracts

The normal lymphoblastoid cell line GM06315A was obtained from the NIGMS Human Genetic Mutant Cell Repository. The Chinese hamster ovary cell lines AA8 and EM9 (Thompson *et al.*, 1982) were obtained from Dr L.Thompson (Lawrence Livermore National Laboratory, CA). Cell-free extracts were prepared by the method of Manley *et al.* (1983) with minor modifications (Wood *et al.*, 1988). Repair reactions (50 μl) contained 45 mM HEPES–KOH (pH 7.8), 70 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dATP, dTTP, dGTP, 2 μM dCTP (Pharmacia), 10 μCi of [α-^{32}P]dCTP (~3000 Ci/mmol; Amersham), 36 μg of BSA, 2% glycerol, substrate DNA (150 fmol) and 20 μg of cell-free extract. Reaction mixtures routinely were supplemented with 1 mM NAD. After incubation at 37°C for between 5 and 60 min, reactions were terminated by addition of SDS to 0.6% and EDTA to 25 mM. Proteinase K was added to 500 μg/ml and tRNA to 400 μg/ml. After further incubation at 37°C for 1 h and extraction with phenol/chloroform, the DNA was recovered by precipitation with ethanol, and analysed by electrophoresis in 20% denaturing polyacrylamide gels and by autoradiography.

Gel retardation assays

Gel retardation assays were performed essentially as described by O'Regan *et al.* (1996). Briefly, proteins (300 fmol) were incubated either singly or together at 20°C for 60 min in 10 μl of reaction buffer containing 40 mM HEPES–KOH (pH 7.8), 70 mM KCl, 1 mM DTT, 0.5 mM EDTA, 7 mM $MgCl_2$, 2 mM ATP, 20 μM each of dATP, dTTP, dGTP and dCTP, 100 μg/ml BSA, 5% glycerol and 5 fmol of ^{32}P-labelled probe DNA (prepared as above), with or without unlabelled competitor DNA. The reaction products were analysed by electrophoresis in 6% polyacrylamide gels as described (Stephenson and Karran, 1989) and were detected by autoradiography.

XRCC1 constructs

Construct δ56–374 (encoding XRCC1 lacking amino acids 56–374) was derived from pET16BHX (Caldecott *et al.*, 1995) by restriction enzyme cleavage and religation. All other constructs were generated from pET16BHX as truncations produced by random transposon insertion of stop codons as described (R.A.Nash, K.W.Caldecott, D.E.Barnes and T.Lindahl, submitted). The site of transposon insertion was determined by DNA sequencing. All constructs were overexpressed in *E coli* BL21(DE3) and purified on Ni-NTA–agarose (Qiagen).

Far Western blotting and affinity precipitation assays

Far Western blotting was performed as described (Caldecott *et al.*, 1995; Wei *et al.*, 1995). Briefly, DNA polymerase β (0.6 μg) was subjected to SDS–PAGE, electroblotted onto nitrocellulose, denatured (using guanidine hydrochloride), renatured and incubated with an XRCC1 probe (1.7 μg) that had been phosphorylated by CKII (Boehringer Mannheim) in the presence of [γ-^{32}P]ATP. After removal of excess probe, blots were dried and analysed by autoradiography.

Affinity precipitation was also performed as described (Caldecott *et al.*, 1995). In brief, histidine-tagged XRCC1 (1–4 μg) was incubated with DNA polymerase β (0.5 μg) and BSA (0.5 μg) at 20°C for 20 min, added to a 25 μl bed volume of Ni-NTA–agarose beads (Qiagen) in 1 mM imidazole (pH 8.0) and incubated for a further 20 min with mixing. After six washes in 105 μl of 25 mM imidazole wash buffer to remove non-specifically associated proteins, 35 μl of 250 mM imidazole elution buffer was added twice to elute histidine-tagged XRCC1 and

bound proteins. Thirty μl of each of the six washes and the two elutions were examined by SDS–PAGE and silver staining of proteins.

Two-hybrid analysis

Full-length human XRCC1 was expressed as a C-terminal fusion to the DNA binding domain (DNA-BD) of the yeast GAL4 transcriptional activator. The fusion construct was generated by cloning the 2.2 kb *Eco*RI fragment from pCD2EHX (Caldecott *et al.*, 1994) into the *Eco*RI site of the DNA-BD vector pAS2-1 (Clontech), which also carries a functional yeast *TRP1* gene. Full-length human DNA polymerase β was expressed as a C-terminal fusion to the activation domain (AD) of GAL4. The expression construct was obtained by cloning the 1 kb *Cla*I–*Hin*dIII fragment from pWL-11 using synthetic double-stranded oligonucleotide adapters into the *Nco*I–*Sma*I sites of the activation vector pACT2 (Clontech), which carries the yeast *LEU2* gene as a selectable marker. The accuracy of cloning steps was confirmed in all cases by DNA sequence analysis. Plasmids pVA3-1 (Clontech), encoding a DNA-BD–murine p53 fusion, and pTD1-1 (Clontech), encoding a AD–SV40 large T antigen fusion, were used as positive controls for β-galactosidase induction. Plasmid pLAM5′-1 (Clontech), encoding a DNA-BD–human lamin C fusion protein, was used in combination with the AD-β-polymerase construct as a negative control.

Combinations of DNA-BD and AD vector constructs (0.5 μg DNA of each) were used for transformation of the yeast strain Y187 (Harper *et al.*, 1993) according to the LiAc method of Gietz *et al.* (1992). Y187 carries the *lacZ* reporter gene under the control of *GAL1* promoter elements and is auxotrophic for leucine (*leu2-3*) and tryptophan (*trp1-901*). Leu^+ Trp^+ co-transformants were selected by plating the cells on synthetic dropout medium lacking leucine and tryptophan (SD –Leu –Trp). After incubation for 4 days at 30°C, large colonies were restreaked on the same medium to isolate individual clones, which were then spread in small patches on SD –Leu –Trp masterplates and grown for 2 days at 30°C. β-Galactosidase activity was assayed qualitatively by lifting the colonies onto Hybond-C-extra filters (Amersham), permeabilizing cells by snap-freezing the filters in liquid nitrogen and thawing at room temperature, and incubating the filters on 3MM Whatman filter paper, pre-soaked in β-galactosidase reaction buffer (100 mM Na phosphate pH 7.0, 10 mM KCl, 1 mM $MgCl_2$, 333 μg/ml X-gal) at 30°C for 2 h.

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