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Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1)

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Two forms of DNA base excision-repair (BER) have been observed: a 'short-patch' BER pathway involving replacement of one nucleotide and a 'long-patch' BER pathway with gap-filling of several nucleotides. The latter mode of repair has been investigated using human cell-free extracts or purified proteins. Correction of a regular abasic site in DNA mainly involves incorporation of a single nucleotide, whereas repair patches of two to six nucleotides in length were found after repair of a reduced or oxidized abasic site. Human AP endonuclease, DNA polymerase β and a DNA ligase (either III or I) were sufficient for the repair of a regular AP site. In contrast, the structure-specific nuclease DNase IV (FEN1) was essential for repair of a reduced AP site, which occurred through the long-patch BER pathway. DNase IV was required for cleavage of a reaction intermediate generated by template strand displacement during gap-filling. XPG, a related nuclease, could not substitute for DNase IV. The long-patch BER pathway was largely dependent on DNA polymerase β in cell extracts, but the reaction could be reconstituted with either DNA polymerase β or δ. Efficient repair of γ-ray-induced oxidized AP sites in plasmid DNA also required DNase IV. PCNA could promote the Pol β-dependent long-patch pathway by stimulation of DNase IV.

Keywords: DNA repair/DNase IV/FEN1/oxidative DNA damage/PCNA

Introduction

DNA base damage generated by ionizing radiation, simple alkylating agents or endogenous hydrolytic and oxidative processes is corrected by the base excision-repair (BER) pathway (reviewed by Seeberg *et al.*, 1995). Repair is initiated by various DNA glycosylases, which excise altered bases by hydrolytic cleavage of the base–sugar bond, generating a regular apurinic/apyrimidinic (AP) site. The deoxyribose residue at a regular AP site is in equilibrium between the furanose form and the free aldehyde form, so the site is susceptible to chain cleavage by β-elimination. Since AP sites in DNA can be produced by non-enzymatic hydrolysis or active oxygen, correction of such base-free sites also occurs by BER. The entire BER process mainly involves replacement of a single nucleotide residue and the reaction has been reproduced *in vitro* using cell-free extracts and purified proteins (Dianov *et al.*, 1992; Singhal *et al.*, 1995; Kubota *et al.*, 1996). However, repair patches are heterogeneous in both bacterial and mammalian systems, with a minority of gap-filling events of up to six nucleotides consistently being observed.

The short-patch BER pathway has been reconstituted with the purified human proteins uracil-DNA glycosylase (UDG), AP endonuclease (HAP1), DNA polymerase β (Pol β), the scaffold protein XRCC1 and either DNA ligase III or DNA ligase I (Kubota *et al.*, 1996). However, the generation of longer repair patches is suppressed by antibodies against another protein, the replication factor 'proliferating cell nuclear antigen' (PCNA), in extracts of Chinese hamster cells (Frosina *et al.*, 1996). In earlier studies with *Xenopus laevis* proteins, Matsumoto *et al.* (1994) showed that long-patch gap-filling at tetrahydrofuran analogues of AP sites in DNA could be achieved with AP endonuclease, Pol δ and PCNA. These results indicate that a distinct repair pathway is responsible for the long-patch gap-filling. To investigate the long-patch type of repair in a human system, DNA substrates with AP sites containing an altered sugar, not amenable to cleavage by β-elimination, were employed. Using such substrates for BER by human cell-free extracts, repair patches were found to span two to six nucleotides. In experiments to reconstitute the complete repair reaction with purified proteins, the structure-specific nuclease DNase IV (FEN1) was found to be required in addition to the proteins necessary for short-patch BER, and PCNA stimulated the reaction.

DNase IV (FEN1) is the mammalian counterpart of the distinct 5′ nuclease domain of *Escherichia coli* DNA polymerase I (Lindahl *et al.*, 1969; Robins *et al.*, 1994), the *Schizosaccharomyces pombe* rad2 protein (Murray *et al.*, 1994) and the *Saccharomyces cerevisiae* Rad27 protein (Reagan *et al.*, 1995). The enzyme recognizes and cleaves 5′ overhang or flap DNA structures (Lundquist and Olivera, 1982; Lyamichev *et al.*, 1993; Harrington and Lieber, 1994a; Robins *et al.*, 1994) and belongs to a family of structure specific nucleases (Harrington and Lieber, 1994b). Moreover, DNase IV has associated 5′→3′ exonuclease and RNase H activities and the enzyme is required during *in vitro* lagging-strand DNA replication (Ishimi *et al.*, 1988; Goulian *et al.*, 1990; Waga *et al.*, 1994; Murante and Bambara, 1995).

Results

Repair of regular and reduced AP sites by human cell extracts

Base excision-repair of DNA containing a uracil residue is performed efficiently by human cell extracts with the generation of a repair patch of a single nucleotide residue (Dianov *et al.*, 1992; Singhal *et al.*, 1995). Such repair is

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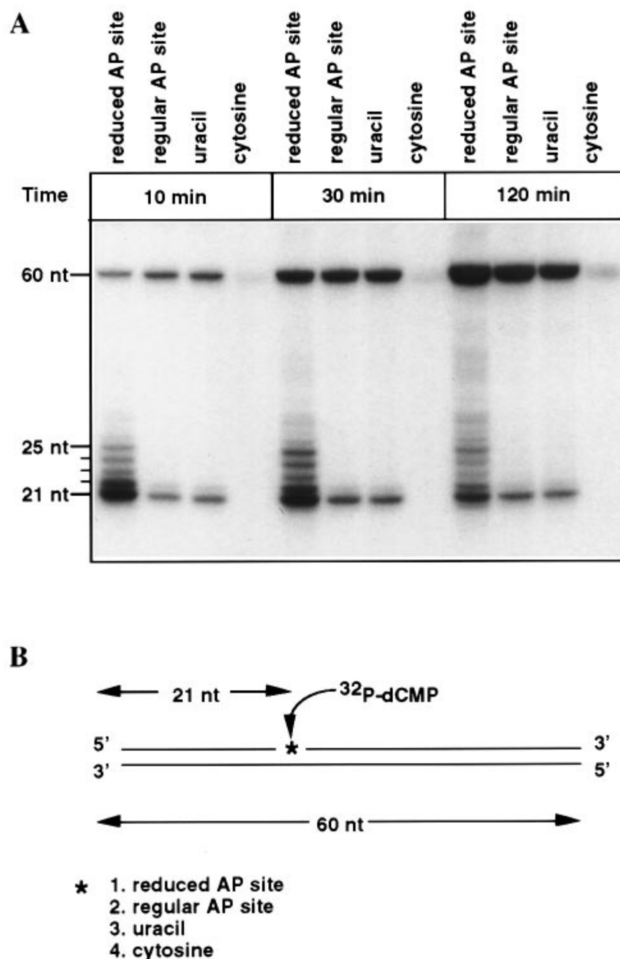

Fig. 1. (**A**) Base excision-repair of double-stranded oligonucleotides containing either a reduced AP site, a regular AP site or a deoxyuridine residue opposite a deoxyguanosine residue by cell extracts from the normal human lymphoblastoid cell line GM06315A. A control substrate with a normal CG pair at the relevant position was also included. Repair reactions were as described in Materials and methods. The type of DNA damage is indicated on top. DNA substrates were incubated with cell extract for 10, 30 or 120 min. Reaction products were analysed by autoradiography after electrophoretic separation in a denaturing 20% polyacrylamide gel. The sizes (nucleotides, nt) and positions of reaction products are indicated; nucleotide residues are numbered from the 5′ end. (**B**) Predicted reaction products. Partial or completed products resulting from replacement of a dUMP with a dCMP residue are shown.

dependent on excision of deoxyribose-phosphate from an incised AP site by the β-elimination (AP lyase) activity of the N-terminal domain of Pol β (Matsumoto and Kim, 1995). A deoxyribophosphodiesterase activity that may serve as a possible back-up has also been detected, but the lability of that enzyme has prevented its extensive purification and characterization (Price and Lindahl, 1991). The AP lyase activity of Pol β is inactive at reduced AP sites. Oligonucleotides with an AP site fully reduced by sodium borohydride treatment can therefore be used as a model substrate for processing of altered AP sites. The ability of cell-free extracts from normal human lymphoblastoid cells (GM06315A) to repair a single 1′-reduced AP site compared with a regular AP site or a dUMP residue in a double-stranded oligonucleotide was tested in a standard reaction mixture (Figure 1). Repair synthesis was monitored by incorporation of [^{32}P]dCMP at the damaged site. Uracil is removed rapidly from the substrate by UDG in the extract, producing a regular AP site. Figure 1 shows that complete DNA repair, with repair synthesis and ligation, could be achieved in cell-free extracts for all tested DNA modifications. The reduced AP site was corrected more slowly than the regular AP site; this could reflect a kinetic barrier for template strand displacement, with suppression of the displacement reaction by the XRCC1 protein (Kubota *et al.*, 1996). Repair synthesis was exclusively dependent upon modifications of DNA; when a cytosine was present instead of a uracil residue, no significant incorporation of radiolabelled dCMP was observed. With a regular AP site the major radiolabelled products were 21mer and 60mer oligonucleotides, showing that one-nucleotide gap-filling occurred, followed by ligation. In contrast, little joining took place at early times with the reduced substrate. In this case, filling-in of the one-nucleotide gap was observed, and also significant template strand displacement synthesis with appearance of repair patches two to six nucleotides long. At later times, the fully repaired oligonucleotide contained larger amounts of [^{32}P]dCMP than the other substrates, indicating that repair of a reduced AP site involved filling of gaps longer than one nucleotide. Identical results were obtained when a 41mer oligonucleotide with a different sequence (see Materials and methods) was used as substrate.

Reconstituted repair of regular and reduced AP sites by purified human proteins

Repair of a double-stranded oligonucleotide containing a centrally placed UG base pair has been reconstituted with purified human proteins (Kubota *et al.*, 1996). The core reaction proceeds as follows: (i) UDG cleaves the dUMP residue leaving a regular AP site; (ii) HAP1 incises the DNA 5′ to the AP site; (iii and iv) Pol β fills the single nucleotide gap and excises the 5′ dRp residue; and (v) the Lig III/XRCC1 heterodimer, or Lig I, seals the gap. By following this scheme, repair of a regular AP site was accomplished here by HAP1, Pol β and Lig I (Figure 2B, lanes 1–3). In contrast, these enzymes were unable to complete repair when an oligonucleotide containing a reduced AP site was used as substrate (Figure 2A, lanes 1–3). Pol β (1 ng) was able to generate repair patches of up to six nucleotides, leading to oligonucleotides of 21–26 nucleotides in length, but no ligated 60mer oligonucleotide product was detected. The longer patches generated by Pol β would be associated with template strand displacement and generation of an overhang or flap structure. Repair of reduced AP sites by a human cell-free extract involved strand displacement synthesis to a similar extent (see Figure 1). DNase IV (FEN1) has the properties of an activity required for removal of the overhang structures that obstruct DNA ligation. In order to establish whether this enzyme was able to remove intermediate splayed arm DNA structures produced during long-patch BER, DNase IV was added to the reconstituted repair mixture. DNase IV is unable to release a 5′ terminal base-free deoxyribose-phosphate residue in free form, whereas it can excise the lesion as part of an oligonucleotide (Price and Lindahl, 1991; De Mott *et al.*, 1996). In agreement with this, addition of DNase IV during repair of a reduced AP site did not significantly stimulate ligation of the 21mer product, but the repair of oligonucleotides 22–26 nucleo-

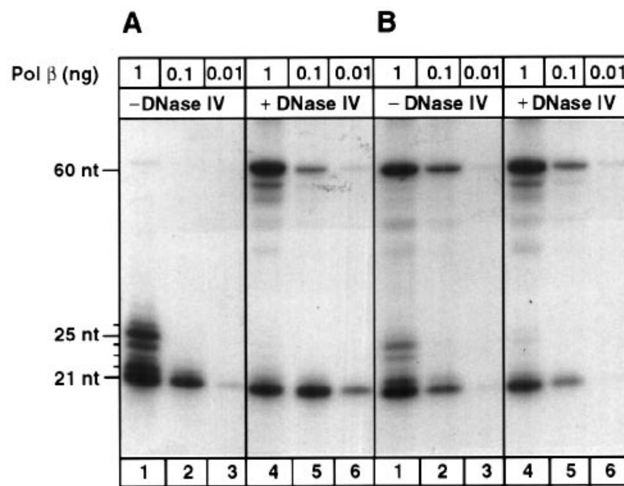

Fig. 2. Reconstituted repair of 1′-reduced AP sites and regular AP sites in DNA by purified human enzymes. Substrates were incubated with HAP1 (10 ng), Lig I (20 ng), increasing amounts of Pol β and either with or without DNase IV (1 ng, ~25 fmol), as indicated. Reaction conditions were as described in Materials and methods. Repair products were analysed by autoradiography after electrophoretic separation in denaturing 20% polyacrylamide gels. Sizes (nucleotides, nt) and positions of reaction products are indicated. (**A**) Substrate containing a 1′-reduced AP site; (**B**) Substrate containing a regular AP site.

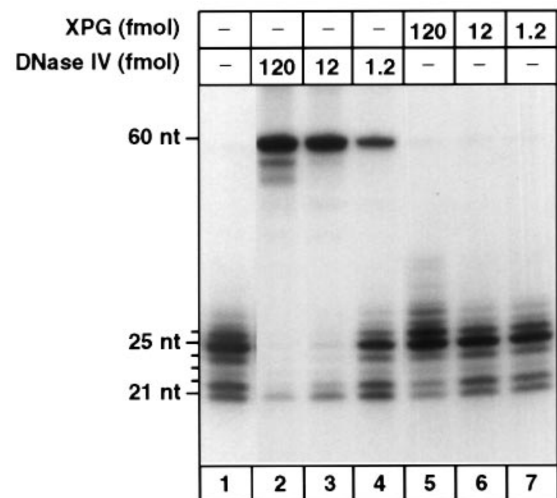

Fig. 3. Attempted substitution of DNase IV with purified XPG nuclease. Reconstituted repair reactions of reduced AP sites were carried out with purified human proteins as in Figure 2A. Results with a reaction mixture containing HAP1 (10 ng), Pol β (2 ng) and Lig I (20 ng) are shown in lane 1. DNase IV was added, as indicated, to the assay mixtures shown in lanes 2, 3 and 4. Corresponding amounts of XPG nuclease were added to the assay mixtures shown in lanes 5, 6 and 7.

tides in length was completed after addition of DNase IV (Figure 2A, lanes 4–6). The addition of DNase IV therefore removed the splayed arm structures produced by excessive gap-filling synthesis by Pol β during repair of the reduced AP site, and also acted on the small amount of such structures produced during repair of a regular AP site (Figure 2B, compare lanes 1 and 4). As observed for the repair reaction with cell extracts, the ligation efficiency increased with extended incubation times (data not shown). In the final joining of the one-nucleotide patch observed during reconstituted repair of a regular AP site, or of the two to six nucleotide repair patch necessary for complete repair of a reduced AP site, human Lig I was able to cooperate with HAP1, Pol β and DNase IV (Figure 2). However, human Lig I could be replaced by human Lig III (added alone or complexed to the XRCC1 protein) and also by phage T4 DNA ligase (data not shown). This lack of specificity for a defined ligase in the *in vitro* BER reactions contrasts with the absolute requirement for Lig I during *in vitro* SV40 lagging-strand DNA replication (Waga *et al.*, 1994; Mackenney *et al.*, 1997).

Inability of XPG to substitute for DNase IV in base excision-repair

The 133 kDa human XPG protein is partly homologous to the 43 kDa human DNase IV, to *S.cerevisiae* Rad27 and Rad2, and to *S.pombe* rad2 and rad13 (Carr *et al.*, 1993). All these structure-specific nucleases recognize and cleave a 5′ branched DNA structure (Harrington and Lieber, 1994b; O'Donovan *et al.*, 1994; Robins *et al.*, 1994; Cloud *et al.*, 1995; Habraken *et al.*, 1995; Evans *et al.*, 1997). Furthermore, XP-G patients are phenotypically one of the most heterogeneous xeroderma pigmentosum complementation groups, indicating a dual function of the XPG protein. To determine whether XPG could remove intermediate splayed arm structures produced during BER of a reduced AP site, and thereby replace DNase IV activity, XPG was added to standard repair reactions. The same concentrations of XPG and DNase IV were employed, whereas the amount of Pol β was twice the amount used in the previously described reactions. At this higher Pol β concentration, a high proportion of strand displaced structures were observed (Figure 3, lane 1). However, XPG was totally unable to produce a suitable substrate for DNA ligation (lanes 5–7). In parallel reconstituted nucleotide excision-repair reactions with bulky DNA lesions, the same batch of XPG protein showed the expected activity (R.D.Wood, personal communication). The highest concentration of XPG used here (lane 5) increased the level of strand displacement; the same effect was apparently also produced by the highest concentrations of DNase IV (lanes 2 and 3), seen as a reduced intensity of the 21 nucleotide intermediate. Complete ligation in the repair reactions was observed with 120 fmol DNase IV (lane 2), and 12 fmol DNase IV also produced $>$95% ligated product (lane 3). The highest concentration of DNase IV employed caused some degradation of the oligonucleotide substrate, probably by the 5′→3′ exonuclease activity of the enzyme.

Human PCNA stimulates the DNase IV-dependent repair pathway

A long-patch BER pathway in vertebrate cell extracts has been reported to be dependent on the abundant nuclear protein PCNA, suggesting involvement of a PCNA-dependent DNA polymerase (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996). However, a stimulatory effect on DNase IV (FEN1) activity by PCNA trimers was described recently, with the proteins forming a 1:1 complex (Li *et al.*, 1995; Wu *et al.*, 1996). This latter finding indicates that PCNA could promote the long-patch BER pathway either by its interaction with DNase IV or with a high-molecular weight DNA polymerase. In order to test the

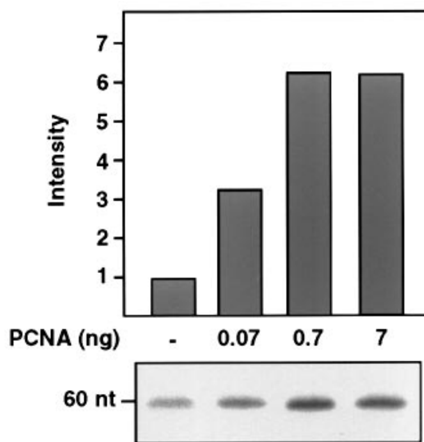

Fig. 4. Stimulation of the DNase IV-dependent long-patch repair pathway by human PCNA. Standard repair reactions containing HAP1 (10 ng), Pol β (1 ng), Lig I (50 ng) and a limiting amount of DNase IV (0.01 ng) were incubated with increasing amounts of PCNA, as indicated. Ligated products were measured by PhosphorImaging, and relative incorporation is shown on the ordinate.

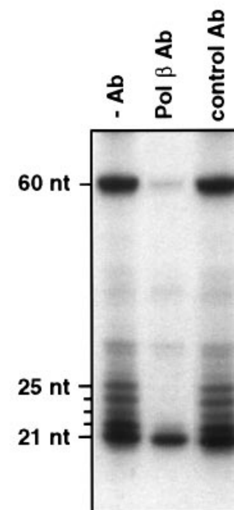

Fig. 5. Inhibition of long-patch BER by antibodies (Ab) against human Pol β. The repair of a reduced AP site by cell extract (as in Figure 1) was carried out without Ab (lane 1), or after 10 min preincubation of cell extract with 50 μg Ab against Pol β (lane 2). Lane 3 shows repair synthesis and ligation after preincubation of the cell extract with 50 μg control Ab against *E.coli* AlkA (3-methyladenine-DNA glycosylase II).

possible functional significance of this stimulation, DNase IV cleavage at 5′ flap structures produced by excessive gap-filling by Pol β was examined, with or without the addition of PCNA (Figure 4). Repair of reduced AP sites using a limiting amount of DNase IV was investigated. Promotion by PCNA of the complete long-patch BER reaction was apparent with 0.07 ng PCNA in the repair reactions. The highest level of stimulation, 6.5-fold, was observed with 0.7 ng PCNA. The requirement for an excess of PCNA over DNase IV probably reflects inefficient DNA loading of PCNA in the absence of a stable replication factor C (RF-C) (Wu *et al.*, 1996).

Inhibition of long-patch repair synthesis by antibodies against Pol β

The identities of DNA polymerases required in different DNA repair pathways remain uncertain. However, strong evidence has been obtained for the involvement of Pol β in the short-patch BER pathway in mammalian cells (Singhal *et al.*, 1995; Nealon *et al.*, 1996; Sobol *et al.*, 1996). Synthesis of DNA by Pol β is distributive on single-stranded DNA templates, but short gaps in double-stranded DNA are filled processively (Singhal and Wilson, 1993). Here, the effect of adding Pol β-specific antibodies to cell-free extracts prior to repair reactions was investigated, using a double-stranded oligonucleotide substrate with a reduced AP site. DNA repair synthesis was decreased 20-fold after preincubation of cell extracts with Pol β neutralizing antibodies (Figure 5, first and second lane). Control antibodies (polyclonal antibodies against *E.coli* AlkA) added at the same concentration had no inhibitory effect on repair synthesis (third lane). The Pol β-specific antibodies do not cross-react with human Pol α, δ or ε, nor with human PCNA (Singhal *et al.*, 1995). Thus, the high-molecular weight Pol δ and ε were inefficient in substituting for Pol β during long-patch BER by a human cell extract in our experimental system.

Pol δ in reconstituted DNA repair of reduced AP sites

A stable RF-C–PCNA complex cannot be assembled on a linear double-stranded oligonucleotide (Podust *et al.*, 1995) and PCNA-promoted repair synthesis by Pol δ or ε might function poorly on such a substrate (Frosina *et al.*, 1996). To test whether Pol δ could perform repair synthesis on a double-stranded oligonucleotide, we reconstituted the reaction for repair of a reduced AP site using Pol δ instead of Pol β. The ability of PCNA and DNase IV to stimulate Pol δ-mediated repair synthesis was tested. In the absence of auxiliary proteins, Pol δ (0.5 U) could fill in a one-nucleotide gap after HAP1 cleavage. Since Pol δ differs from Pol β in not having AP lyase activity, the 5′ deoxyribose-phosphate residue cannot be removed, and the reaction intermediate would not be ligatable. In agreement with this notion, no effect of Lig I addition was detected (Figure 6, lanes 1 and 5). Addition of PCNA to the repair reaction stimulated strand displacement by Pol δ (lanes 2 and 6). Some full-length products, not dependent upon the addition of Lig I, were detected and probably reflected processive synthesis to the end of the double-stranded oligonucleotide substrate by Pol δ. Addition of DNase IV, in the absence of PCNA, had no significant effect on the repair reaction (lanes 3 and 7). Displacement of the HAP1-cleaved strand, resulting from excessive gap-filling by Pol δ, was greatly stimulated when both PCNA and DNase IV were added to the reaction mixture (lane 4). Patches of two or more nucleotides in length, leading to labelled oligonucleotides of 22–27 nucleotides, were efficiently joined by adding Lig I (lane 8) or Lig III (not shown). When both DNase IV and PCNA were added to the reaction mixture, efficient repair was achieved with as little as 0.05 U Pol δ (data not shown). These results show that a combination of PCNA and DNase IV promotes repair with patches two to six nucleotides long involving Pol δ repair synthesis. As found for Pol β-stimulated long-patch BER synthesis, DNase IV was required for complete repair.

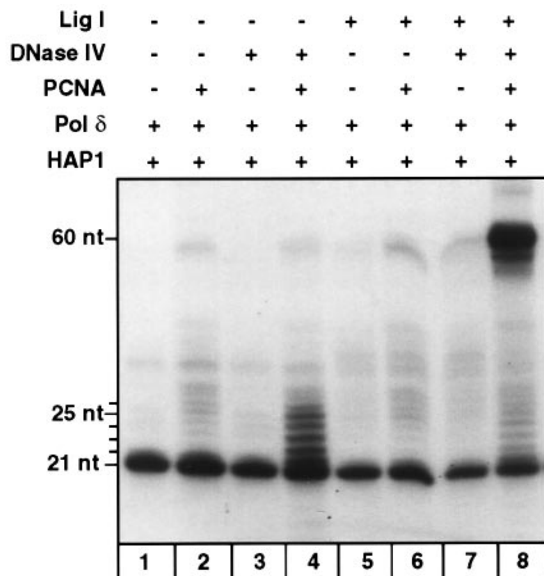

Fig. 6. Reconstituted repair of reduced AP sites by Pol δ. DNA oligonucleotide substrates containing a 1′-reduced AP site were incubated (as indicated) with: HAP1 (10 ng), Pol δ (0.5 U), PCNA (2 ng), DNase IV (1 ng) and Lig I (20 ng). Repair synthesis was monitored by the incorporation of [^{32}P]dCMP, and reaction conditions were as described in Materials and methods. Repair products were analysed by autoradiography after electrophoretic separation in 20% denaturing polyacrylamide gels. Sizes and positions of reaction products are indicated.

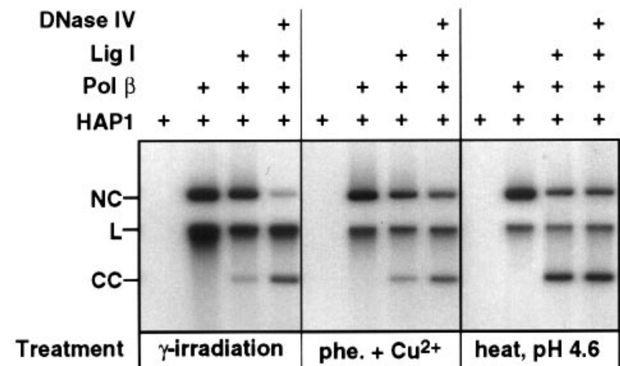

Fig. 7. Repair of AP sites in pBluescript plasmids by purified human proteins. Plasmid DNA was treated with γ-irradiation, phenanthroline/Cu^{2+} or heating at pH 4.6, as described in Materials and methods. The ability of different combinations of human proteins HAP1 (10 ng), Pol β (1 ng), Lig I (20 ng) and DNase IV (1 ng) to perform repair synthesis and ligation were tested. Repair products were separated by 1% agarose gel electrophoresis and analysed by autoradiography. The conformations of plasmid DNA are indicated; NC = nicked circular, L = linear, CC = closed circular.

Reconstituted repair of damaged plasmid DNA

The modified AP site used in the experiments described above was produced by $NaBH_4$ reduction of a regular AP site. *In vivo*, a range of different AP site species in DNA may be produced by exposure to hydroxyl radicals. In order to establish whether the DNase IV-dependent, long-patch BER pathway is required for repair of oxidized AP site species, we assayed the ability of the human enzymes HAP1, Pol β and Lig I, with or without the addition of DNase IV, to repair such AP sites introduced into plasmid DNA. AP sites were generated by treating pBluescript DNA either with (i) γ-irradiation, by which initial hydrogen abstraction at C1, C2 and C4 of sugar moieties occurs (Povirk and Steighner, 1989; Breen and Murphy, 1995); (ii) incubation with phenanthroline/Cu^{2+}, which causes predominantly hydroxylation at C1 (Meunier, 1992); or as a control (iii) heat treatment (below T_m) at pH 4.6. This last procedure causes hydrolytic loss of purines, producing regular AP sites (Lindahl and Nyberg, 1972). Reaction conditions were as for reconstituted repair of DNA oligonucleotides, except that [α-^{32}P]dATP was used to monitor repair synthesis. Repair of γ-irradiated and phenanthroline-treated plasmid DNA to a closed circular (CC) form was clearly improved by the presence of DNase IV in the repair reaction (Figure 7). The amount of CC DNA formation during repair of AP sites introduced by γ-irradiation was promoted 5.1-fold when DNase IV was added to reaction mixtures, whereas repair of phenanthroline/Cu^{2+}-induced AP sites was stimulated 3.2-fold. The conversion of heat-treated plasmid, cleaved at AP sites by HAP1, to CC DNA was not promoted significantly by DNase IV. This is in agreement with results for the repair of regular AP sites in double-stranded oligonucleotide substrates in similar reaction conditions (see Figure 2B, lanes 1 and 4).

Discussion

Exposure of DNA to active oxygen causes the formation of several different lesions, including oxidized AP sites (Demple and Harrison, 1994). These sites can be incised by hydrolytic AP endonucleases, but subsequent excision of the altered sugar-phosphate residue is refractory to β-elimination. In consequence, such altered AP sites cannot be repaired by Pol β in the same way as regular AP sites in mammalian cells. However, complete repair of altered AP sites can be achieved *in vitro* by inclusion of the structure-specific nuclease DNase IV (FEN1) in reaction mixtures, together with the enzymes HAP1, Pol β and a DNA ligase. The latter factors are also required for repair of regular AP sites by replacement of a single nucleotide. Since DNase IV only can release a sugar-phosphate residue as part of an oligonucleotide, longer repair patches are produced when the nuclease is involved. The reaction is promoted by the abundant replication factor PCNA. This factor specifically binds to, and enhances the activity of DNase IV (Li *et al.*, 1995; Wu *et al.*, 1996). The present *in vitro* data indicate that the interaction between DNase IV and PCNA may account for the stimulatory effect of PCNA. In other studies on this pathway (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996), it has been assumed that the PCNA stimulation reflected involvement of Pol δ rather than Pol β in the DNA gap-filling process. Whereas either Pol β or Pol δ could perform DNA repair synthesis in the present reconstituted system, Pol β antibodies strongly suppressed the repair reaction in cell extracts.

The involvement of the replication factors DNase IV and PCNA in the long-patch BER of oxidized or reduced AP sites suggests that completion of repair of these lesions might occur largely at specific sites in the cell nucleus associated with replication. Immunofluorescence studies on the nuclear redistribution of PCNA after exposure of cells to ionizing radiation (Miura *et al.*, 1996) supports

this model. Such a localized mode of repair could also result in an increased dependence on other replication enzymes, e.g. Pol δ or Lig I, for the long-patch BER process, although we did not observe specificity for these factors in the soluble extracts and reconstituted reaction mixtures employed here, where Pol β and Lig III functioned similarly well during gap-filling and ligation.

DNA-damaging agents such as ionizing radiation and alkylating agents can generate AP sites and single-strand breaks in DNA, and the AP sites are incised by HAP1. The abundant nuclear enzyme poly(ADP-ribose) polymerase (PARP) binds rapidly and tightly to such DNA single-strand interruptions, and may cause a brief delay in BER (Satoh *et al.*, 1994). In contrast, DNA-dependent protein kinase binds poorly to single-strand breaks, but is activated by double-strand breaks (Gottlieb and Jackson, 1993). DNA binding of PARP triggers extensive automodification by poly(ADP-ribose) synthesis, which serves to liberate PARP from DNA by charge repulsion (Zahradka and Ebisuzaki, 1982). The poly(ADP-ribose) synthesis at sites of DNA damage may also have an anti-recombinogenic effect, suppressing genomic instability by temporary prevention of the formation of recombination intermediates (Satoh *et al.*, 1994). Mice deficient in PARP exhibit normal resistance to alkylating agents, which strongly indicates that PARP is not directly involved in the BER process (Wang *et al.*, 1995). However, the XRCC1 scaffold protein, which binds both Pol β and Lig III and is involved in short-patch BER and perhaps also long-patch BER, has been shown to have a third distinct binding site for PARP (G.de Murcia and J.de Murcia, personal communication). It seems likely that PARP, through its interaction with XRCC1, can recruit the BER machinery to strand interruptions in DNA, which would allow repair of the damaged site in preference to binding of additional PARP molecules. In cells lacking PARP, the BER process would still proceed as normal, since PARP is not an essential component of this repair pathway.

The present data provide additional evidence for the existence of two distinct pathways for completion of BER in vertebrates (Matsumoto *et al.*, 1994; Frosina *et al.*, 1996). A current model is shown in Figure 8. The short-patch pathway is the dominant route in mammalian cells. In contrast, BER in *S.cerevisiae* occurs mainly or entirely by the long-patch pathway (Wang *et al.*, 1993; Blank *et al.*, 1994). The reconstitution of the long-patch BER pathway with purified human enzymes allows for further studies on potential accessory repair factors.

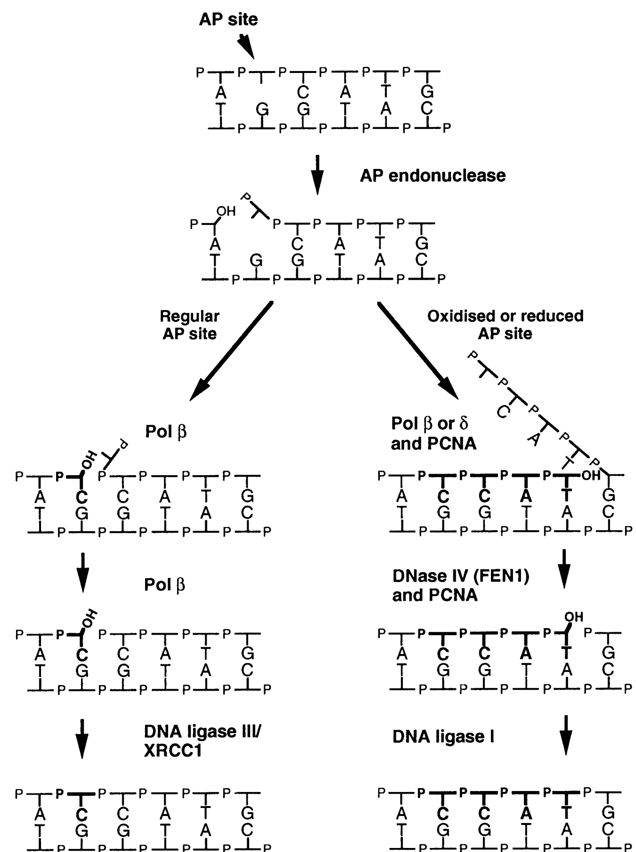

Fig. 8. Branched pathway for repair of AP sites in DNA by human enzymes. The proteins believed to be responsible for the various reaction steps are indicated. In the left branch, a single nucleotide repair patch will be formed, whereas in the right branch two to six nucleotides are replaced. Regular AP sites are usually repaired by the short-patch pathway, but a small proportion of such sites may be repaired by the long-patch pathway; in contrast, oxidized and reduced AP sites are refractory to repair by the short-patch pathway and can only be processed by the long-patch pathway. Repair synthesis is in bold.

Materials and methods

Cell extracts and recombinant human enzymes

Cell extracts were prepared by the method of Manley *et al.* (1980) with minor modifications (Wood *et al.*, 1995) from the normal human lymphoblastoid cell line GM06315A (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ). Recombinant DNase IV/FEN-1 was provided by R.Savva and L.Pearl (Department of Crystallography, Birkbeck College, London, UK), uracil-DNA glycosylase (UDG) by H.Krokan (Slupphaug *et al.*, 1995), human apurinic/apyrimidinic endonuclease (HAP1) by I.Hickson (Walker *et al.*, 1993) and Pol δ by U.Hübscher (Podust *et al.*, 1995). Human Pol β was expressed from plasmid pWL-11 (from S.Wilson) and purified as described (Abbotts *et al.*, 1988). Recombinant XPG nuclease and PCNA were provided by R.Wood (Shivji *et al.*, 1992; O'Donovan *et al.*, 1994). Active recombinant DNA ligase I with an N-terminal histidine tag, and recombinant DNA ligase III/XRCCI heterodimer were purified as described (Mackenney *et al.*, 1997; Nash *et al.*, 1997). *E.coli* endonuclease III was purified from an overexpressing strain (Asahara *et al.*, 1989).

DNA and oligonucleotide substrates

Single-stranded oligonucleotides containing a U residue were prepared on a commercial DNA synthesizer, purified on a denaturing 20% polyacrylamide sequencing gel and annealed to complementary oligonucleotides with a G residue opposite the U residue. All oligonucleotides included phosphorothioate linkages at the ultimate and penultimate 5′ and 3′ ends to reduce exonucleolytic attack. The 60mer oligonucleotide used was 5′-TAGACATTGCCCTCGAGGTA**U**CATGGATCCGATTT-CGACCTCAAACCTAGACGAATTCCG-3′ and the 41mer 5′-TAGA-CGGATGAATATTGAGG**U**CAGAAGTTGGATTTGGTAGT-3′. Oligonucleotides with a cytosine replacing the uracil residue were also synthesized and used as controls. AP sites were produced by incubating single-stranded uracil-containing oligonucleotides with human UDG. When indicated, oligonucleotides with AP sites were treated with $NaBH_4$ to generate reduced AP sites (Hadi and Goldthwait, 1971). Briefly, potassium phosphate buffer (pH 6.5) was added to oligonucleotides to 0.5 M, and freshly prepared $NaBH_4$ added in three aliquots at 15 min intervals to a final concentration of 0.25 M. The solution was incubated at room temperature for 1 h. After treatment, oligonucleotides were dialysed for 2 h against 10 mM Tris–HCl, 1 mM EDTA, pH 7.5 (TE) and purified on a 20% denaturing polyacrylamide sequencing gel. For annealing of complementary strands, single-stranded oligonucleotides were incubated for 2 min at 90°C in TE and allowed to cool slowly to 20°C. *E.coli* endonuclease III was unable to cleave double-stranded

DNA oligonucleotides treated with $NaBH_4$, indicating that all regular AP sites had been converted to 1′-reduced AP sites.

Plasmid pBluescript KS^+ (Stratagene) was purified from the *E.coli* DH5α host strain using a Maxi-plasmid purification kit (Qiagen Ltd). For γ-irradiated plasmid DNA, pBluescript (800 μg/ml in TE pH 8.0) was exposed to a ^{137}Cs γ-ray source at 2.8 Gy/min (total dose 100 Gy) at 0°C. Covalently closed circular plasmid DNA was isolated by ethidium bromide/CsCl density gradient centrifugation and dialysed for 20 h with two changes of TE. For phenanthroline-Cu(II) treatment, pBluescript KS^+ (15 μg/ml), in 50 mM NaCl, 5 mM potassium phosphate (pH 7.4), 5% ethanol, was incubated for 30 min at 25°C in the presence of 1 μM $CuSO_4$ and 4 μM phenanthroline. The reaction was initiated by the addition of H_2O_2 to 0.5 mM, and terminated by dilution with 2 vols of TE (pH 8.0). The plasmid was immediately concentrated and washed with 3 vols of TE using a Centricon C100. Regular AP sites were introduced into pBluescript KS^+ by heating the plasmid DNA (15 μg/ml) in 0.1 M NaCl, 10 mM NaH_2PO_4, 10 mM citric acid, pH 4.6, for 10 min at 70°C. The reaction was terminated by cooling on ice, and the DNA was washed and concentrated as above. On incubation with the AP site specific HAP1 nuclease, 60–80% of treated plasmids were nicked.

DNA repair reactions

Standard reaction mixtures (50 μl) contained 150 fmol double-stranded oligonucleotide substrate, 45 mM HEPES–KOH pH 7.8, 70 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.4 mM EDTA, 2 mM ATP, 1 mM NAD, 20 μM each of dATP, dGTP and dTTP, 2 μM dCTP, 10 μCi [α-^{32}P]dCTP (~3000 Ci/mmol), 36 μg BSA, 2% glycerol and 15 μg cell extract. Reactions were incubated at 37°C for 30 min and terminated by addition of SDS to 0.6% and EDTA to 25 mM. Proteinase K was then added to 500 μg/ml. After digestion for 1 h at 37°C and extraction with phenol/chloroform, tRNA carrier was added to 0.4 mg/ml and the oligonucleotide material was ethanol precipitated. This substrate was resolved by denaturing 20% polyacrylamide gel electrophoresis and visualized by autoradiography or analysed on a PhosphorImager for quantification (Molecular Dynamics). For the reconstituted DNA repair reactions with purified enzymes, the same conditions were used as described above, but the reaction volume was reduced to 10 μl, the amount of DNA oligonucleotide substrate kept at 150 fmol, and NAD was not added. Repair of damaged plasmid DNA by human enzymes was as for the repair of synthetic oligonucleotides, but each reaction mixture contained 100 ng plasmid DNA.

Antibodies

Neutralizing polyclonal rabbit antibodies raised against human DNA Pol β and purified by affinity chromatography to coupled Pol β, were provided by S.H.Wilson (Singhal *et al.*,1995). Control antibodies were similarly purified (Bjørås *et al.*, 1995). To inhibit Pol β activity, cell extracts (15 μg protein) were preincubated with 50 μg antibodies for 10 min at 4°C prior to carrying out DNA repair reactions.

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