

# The Type of DNA Glycosylase Determines the Base Excision Repair Pathway in Mammalian Cells\*

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The base excision repair (BER) of modified nucleotides is initiated by damage-specific DNA glycosylases. The repair of the resulting apurinic/aprimidinic site involves the replacement of either a single nucleotide (short patch BER) or of several nucleotides (long patch BER). The mechanism that controls the selection of either BER pathway is unknown. We tested the hypothesis that the type of base damage present on DNA, by determining the specific DNA glycosylase in charge of its excision, drives the repair of the resulting abasic site intermediate to either BER branch. In mammalian cells hypoxanthine (HX) and 1,*N*<sup>6</sup>-ethenoadenine ( $\epsilon$ A) are both substrates for the monofunctional 3-methyladenine DNA glycosylase, the ANPG protein, whereas 7,8-dihydro-8-oxoguanine (8-oxoG) is removed by the bifunctional DNA glycosylase/ $\beta$ -lyase 8-oxoG-DNA glycosylase (OGG1). Circular plasmid molecules containing a single HX,  $\epsilon$ A, or 8-oxoG were constructed. *In vitro* repair assays with HeLa cell extracts revealed that HX and  $\epsilon$ A are repaired via both short and long patch BER, whereas 8-oxoG is repaired mainly via the short patch pathway. The preferential repair of 8-oxoG by short patch BER was confirmed by the low efficiency of repair of this lesion by DNA polymerase  $\beta$ -deficient mouse cells as compared with their wild-type counterpart. These data fit into a model where the intrinsic properties of the DNA glycosylase that recognizes the lesion selects the branch of BER that will restore the intact DNA template.

Various DNA-damaging agents produce modified bases in DNA that are repaired by the base excision repair (BER)<sup>1</sup> pathway (reviewed in Ref. 1). A number of DNA glycosylases recognize the damaged bases and remove them through *N*-glycosidic bond hydrolysis. There are two types of DNA-glycosylases: the monofunctional exhibiting only the glycosylase activity and the bifunctional, which are glycosylase/ $\beta$ -lyases. In human cells, an example of the first category is the 3-methyladenine-DNA glycosylase, the ANPG protein. It catalyzes the

excision of a broad variety of modified bases including *N*-methylpurines generated by alkylating agents (reviewed in Ref. 1), deamination products like hypoxanthine (HX) (2), and 1,*N*<sup>6</sup>-ethenoadenine ( $\epsilon$ A), an adduct generated by chloroacetaldehyde or products of lipid peroxidation (3, 4). An example of a DNA glycosylase with an associated AP-lyase is hOGG1, the human homolog of the *Escherichia coli* Fpg protein (formamidopyrimidine-DNA glycosylase) (5), which excises the potent premutagenic lesion 7,8-dihydroxy-8-oxoguanine (8-oxoG) (6–11). The removal of 8-oxoG is followed by DNA strand cleavage by hOGG1 via  $\beta$ -elimination (reviewed in Ref. 1).

This damage-specific initial step, carried out by individual DNA glycosylases, is followed by the processing of the resulting apurinic/aprimidinic (AP) site, a mutagenic repair intermediate (reviewed in Ref. 12), presumably by the major mammalian AP-endonuclease, HAP1/APEX (reviewed in Ref. 13). AP sites are processed via two alternative pathways: the short patch (1-nucleotide gap filling) (14–16) and the long patch (2–6 nucleotide resynthesis) BER (17–19). These two pathways involve some common proteins but also some specific ones. For example in the short patch pathway, DNA polymerase (Pol)  $\beta$  is involved in the resynthesis step (20), whereas PCNA and Pol  $\delta/\epsilon$  are implicated in the long patch pathway (21). It therefore becomes important to investigate whether the initial recognition step of the modified base by a specific DNA glycosylase targets the following repair steps to a specific BER branch. In this study, specific lesions for the two types of DNA-glycosylases, namely HX and  $\epsilon$ A for the monofunctional ANPG protein and 8-oxoG for the bifunctional OGG1 protein were selected, and their respective DNA repair pathways were analyzed. Circular duplex plasmids containing a single lesion at a known position of their genome were used as DNA substrates to distinguish the two BER branches by fine mapping of the repair synthesis patches. We report that HX and  $\epsilon$ A are repaired via the short as well as the long patch BER, whereas the 8-oxoG residues are preferentially repaired via the short patch pathway. Because Pol  $\beta$  is involved in the short patch repair, we show, as expected, that Pol  $\beta$ -null mouse cell extracts exhibited a significantly slower repair of 8-oxoG residues as compared with their wild-type counterpart.

## EXPERIMENTAL PROCEDURES

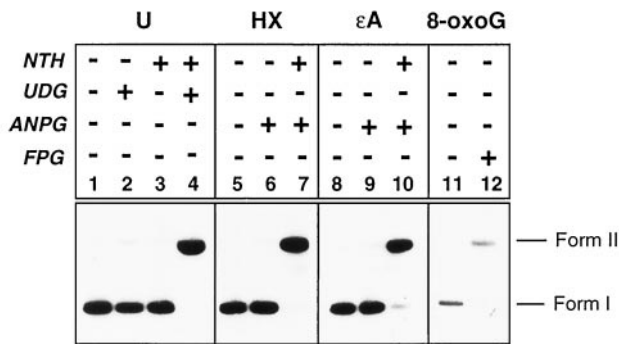
**Chemicals, Enzymes, and Cell-free Extracts**—Chemicals were purchased from Sigma, and molecular biological reagents were from Roche Molecular Biochemicals or New England Biolabs. [ $\alpha$ -<sup>32</sup>P]dATP, [ $\alpha$ -<sup>32</sup>P]dCTP, [ $\alpha$ -<sup>32</sup>P]dTTP, and [ $\alpha$ -<sup>32</sup>P]dGTP (3000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. T4 DNA polymerase holoenzyme, single-stranded DNA binding protein and T4 DNA ligase were purchased from Roche Molecular Biochemicals.

For the oligodeoxyribonucleotides containing a single uracil, HX,  $\epsilon$ A, or 8-oxoG residue, 5'-GATCCTCTAGAGUCGACCTGCA-3' and 5'-GATCCTCTAGAGTTCG(HX)CCTGCAGGCATGCA-3' were synthesized by M-Medical (Florence, Italy), and 5'-GATCCTCTAGAGTTCG( $\epsilon$ A)CCTGCAGGCATGCA-3' was synthesized by Genset (Paris, France)

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<sup>1</sup> The abbreviations used are: BER, base excision repair; NER, nucleotide excision repair; AP, apurinic/aprimidinic; Pol  $\beta$ ,  $\delta$ , and  $\epsilon$ , DNA polymerase  $\beta$ ,  $\delta$ , and  $\epsilon$ ; HX, hypoxanthine;  $\epsilon$ A, 1,*N*<sup>6</sup>-ethenoadenine; 8-oxoG, 7,8-dihydro-8-oxoguanine; bp, base pair; PCNA, proliferating cell nuclear antigen; ANPG, alkyl *N* purine glycosylase.



**FIG. 1. Characterization of single lesion containing substrates.** Lane 1, construct containing a single uracil residue (U); lane 2, after incubation with UDG; lane 3, after incubation with Nth; lane 4, after incubation with UDG followed by incubation with Nth; lane 5, construct containing a single HX; lane 6, after incubation with APNG; lane 7, after incubation with APNG followed by incubation with Nth; lane 8, construct containing a single εA; lane 9, after incubation with APNG; lane 10, after incubation with APNG followed by incubation with Nth; lane 11, construct containing a single 8-oxoG; lane 12, after incubation with Fpg.

and 5'-GATCCTCTAGAGTC(8oxoG) ACCTGCAGGCATGCA-3' was synthesized by Eurogentec (Angers, France). These oligonucleotides were used to create duplex plasmid molecules containing a single lesion at a defined position.

The recombinant Nth protein (endonuclease III), ANPG, and Fpg proteins were purified to homogeneity as described in Refs. 4, 5, and 22. *E. coli* uracil-DNA glycosylase, UDG protein, was a gift of Dr. S. Boiteux (Centre Energie Atomique, Fontenay aux Roses, France). Purified recombinant rat Pol β was a gift of Dr. J. S. Hoffmann (CNRS, Institut de Pharmacologie et Biologie Structurale, Toulouse, France). Whole cell extracts from HeLa cells and wild-type and Pol β-null mouse fibroblasts (a gift of Dr. S. H. Wilson, NIEHS, Research Triangle Park, NC) were prepared as described previously (18, 23). To separate PCNA from other repair proteins, whole cell extracts were chromatographed onto a phosphocellulose column as described (24). Under these experimental conditions, the flow-through fraction (CFI) contained PCNA, and the bound fraction (CFII) contained all proteins essential for short patch BER. We have verified that the addition of PCNA to CFII is required to observe the long patch BER.<sup>2</sup>

**Preparation of DNA Substrates**—Closed circular DNA containing a single lesion was produced as described previously (18) by priming single-stranded (+) pGEM-3Zf DNA (Promega) with the oligonucleotide containing the modified base of interest. It was further incubated with T4 DNA polymerase holoenzyme, single-stranded DNA binding protein, dNTPs, and T4 DNA ligase. Closed circular DNA duplex molecules were purified by cesium chloride equilibrium centrifugation. The plasmid DNA containing a single uracil residue was digested with UDG to produce a single abasic site. The oligonucleotide 5'-GATCCTCTAGAGTCGACCTGCA-3' was used to prepare the control plasmid.

**In Vitro Repair Assays**—Repair reactions were carried out essentially as described in Ref. 18. Briefly, reaction mixtures (50 μl) contained 40 mM Hepes/KOH (pH 7.9), 75 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 20 μM of each dNTP, 2 μCi of [α-<sup>32</sup>P]dATP, [α-<sup>32</sup>P]dCTP, [α-<sup>32</sup>P]dTTP, or [α-<sup>32</sup>P]dGTP as indicated, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg of creatine phosphokinase (type I, Sigma), 3.4% glycerol, 18 μg of bovine serum albumin, and 20 μg of human cell extracts. After increasing periods of time at 30 °C, the plasmid DNA was recovered and digested with restriction enzymes as indicated. The digestion products were electrophoresed on a denaturing 15% polyacrylamide gel. The repair products were visualized by autoradiography and quantified by electronic autoradiography (Instant Imager, Packard).

## RESULTS

**Characterization of the DNA Substrates Containing Different Lesions**—Single-stranded (+) pGEM-3Zf DNA was primed

with 5'-end <sup>32</sup>P-labeled oligonucleotide containing the specific modified base, and DNA was synthesized *in vitro* using this primed template. As shown in Fig. 1, in all cases closed circular molecules (Form I) were obtained with an efficiency close to 100% (lanes 1, 5, 8, and 11). When the duplex DNA molecules containing the lesion were digested with the specific monofunctional DNA-glycosylase, namely the UDG protein in the case of the uracil residue (lane 2) and ANPG protein in the case of HX (lane 6) and εA (lane 9) lesions, followed by Nth protein (that incises DNA at AP sites by a β-elimination mechanism), a complete conversion of Form I to Form II molecules was observed (lanes 4, 7, and 10). In the case of the plasmid containing the 8-oxoG residue (lanes 11 and 12), the incubation with the Fpg protein was sufficient *per se* to convert supercoiled plasmids to nicked circular forms because this enzyme is also endowed with an AP-lyase activity that incises at AP sites by a β-δ elimination mechanism (25, 26). The recombinant plasmid molecules were partially or fully resistant to cleavage by restriction enzymes (*SalI*, *AccI*, and *HincII*) whose recognition sequence include the modified base confirming that a single lesion was precisely inserted at the expected position of the plasmid genome (data not shown). These data show that these duplex DNA molecules are homogeneous closed circular molecules all containing a single modified base.

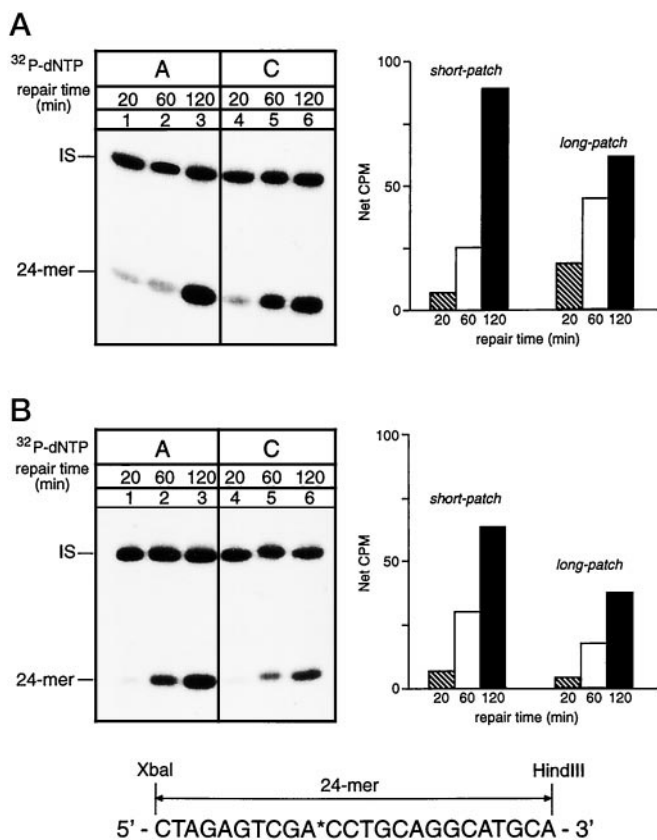
**HX and εA Are Processed via Both Short and Long Patch BER**—To distinguish between the short and long patch BER pathways, the experimental approach involves the use of different labeled dNTPs in the reaction mixture. The incorporation of the specific radiolabeled dNTP at the nucleotide position where the lesion was originally located within the *XbaI-HindIII* restriction fragment (24 nucleotides) marks mainly the occurrence of one-gap filling reactions, whereas the incorporation of dCMP identifies long repair patches (Fig. 2, see *scheme at bottom*) (27). Moreover, because PCNA is required for the long patch but not for the short patch BER (18), the dependence of the repair reaction on this auxiliary protein is an additional marker to distinguish between the two pathways.

The deamination of adenine residues in DNA generates HX, whereas lipid peroxidation generates exocyclic adducts and among them εA. Both HX and εA are repaired, in human cells, by the same gene product, the ANPG protein. As shown in Fig. 2, HeLa cell extracts were proficient in the repair of these modified bases via both pathways: replacement of one nucleotide (Fig. 2, *A* and *B*, lanes 1–3) and resynthesis of longer patches (Fig. 2, *A* and *B*, lanes 4–6). Besides the radiolabeled 24-mer, which is a marker of fully repaired DNA, the other radioactively labeled DNA present on the gel autoradiography is a 5' end-labeled 60-mer internal standard added in all reaction mixtures. This internal standard is used to correct the repair incorporation values (as measured by electronic autoradiography; Fig. 2, *right panels*) for DNA recovery.

A comparison of the nature and the relative contribution of the two BER pathways when using plasmids containing either HX or εA with the repair of preformed AP site showed that all three lesions were repaired by the same pathways with a comparable efficiency (data not shown), suggesting that the AP site processing could be the rate-limiting step in the repair initiated by the ANPG protein.

**8-oxoG Is Repaired Preferentially via Short Patch BER**—In mammalian cells 8-oxoG is excised from oxidatively damaged DNA by the enzyme OGG1, which excises 8-oxoG residue when paired with cytosine and then nicks DNA next to the AP site (28). Fig. 3A shows that crude HeLa cell extracts were able to repair this oxidized guanine *in vitro* but mainly via the short patch BER (lanes 1–3). DNA repair patches longer than 1–2 nucleotides were undetectable on the gel autoradiography after

<sup>2</sup> B. Pascucci and E. Dogliotti, unpublished data.

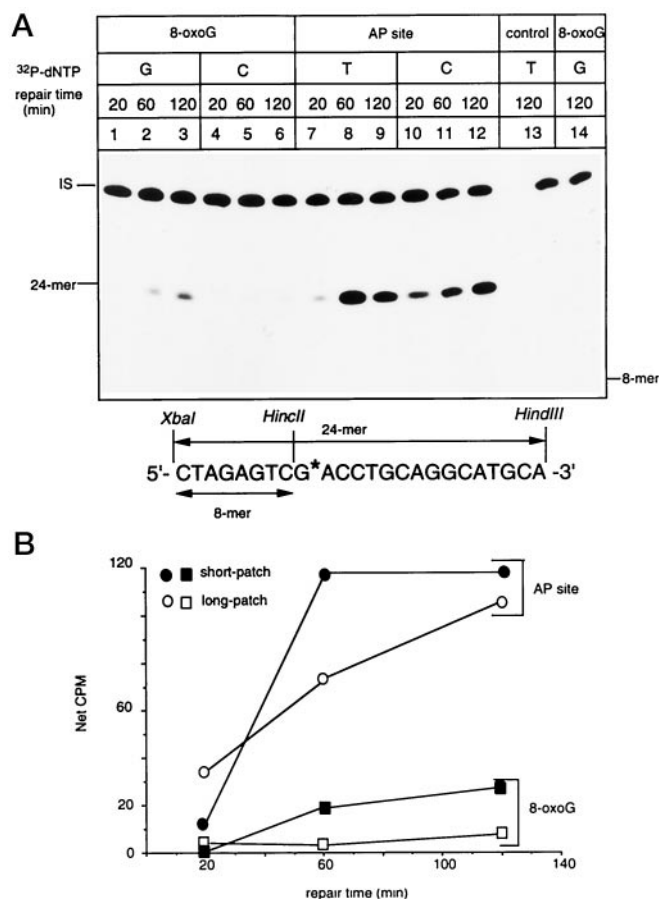


**FIG. 2. Repair of HX and eA by HeLa cell extracts.** Repair replication was performed for different periods of time in the presence of [ $\alpha$ -<sup>32</sup>P]dATP or [ $\alpha$ -<sup>32</sup>P]dCTP as indicated. The plasmid DNA was then digested with *Xba*I-*Hind*III to release the 24-bp fragment originally containing the lesion. *A*, left, autoradiograph of a denaturing polyacrylamide gel showing repair synthesis at the HX residue. Lanes 1–3, short patch repair synthesis as a function of the incubation time; lanes 4–6, long patch repair synthesis as a function of the incubation time. *B*, left, autoradiograph of a denaturing polyacrylamide gel showing repair synthesis at the eA residue. Lanes 1–3, short patch repair synthesis as a function of the incubation time; lanes 4–6, long patch repair synthesis as a function of the incubation time. *A* and *B*, right, the repair products were measured by electronic autoradiography and relative incorporation, corrected for DNA recovery, is indicated on the ordinate (Net CPM).

up to 2 h of repair time (lanes 4–6). The absence of significant incorporation of dCMP into the repair fragment indicates that 8-oxoG is a poor substrate for long patch BER, at least extending beyond two nucleotides. Moreover, it also strongly suggests that nucleotide excision repair (NER) is barely or not involved in the repair of this lesion under our experimental conditions. This fact is confirmed by the lack of repair synthesis in the *Xba*I-*Hinc*II digestion fragment (lane 14), which includes 7 nucleotides 5'-flanking the lesion.

The comparison of the extent of repair of the 8-oxoG lesion (lanes 1–6) with that of an AP site constructed in the same circular plasmid (lanes 7–12) showed that the repair activity toward an AP site was significantly higher than the specific repair of 8-oxoG residues (Fig. 3B). This result suggests that in the repair of oxidative DNA damage, the efficiency of the initial step(s), up to the production of 3' OH primers for repair synthesis, determines the overall repair activity.

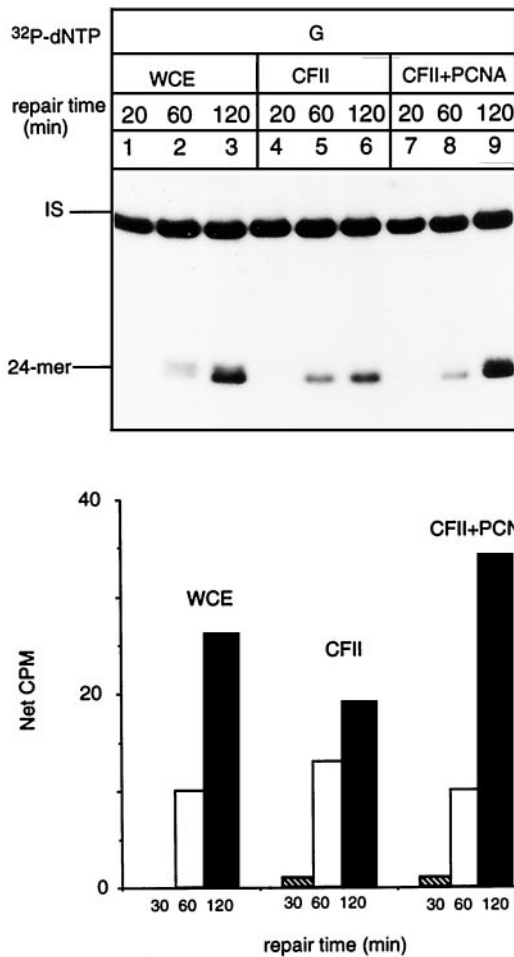
**Short Patch BER of 8-oxoG Is PCNA-independent and Involves Mainly the Replacement of a Single Nucleotide**—PCNA is not only a protein required for DNA replication but also plays a key role in long patch BER and NER (reviewed in Ref. 29). A purified HeLa cell extract fraction, CFII, containing all the



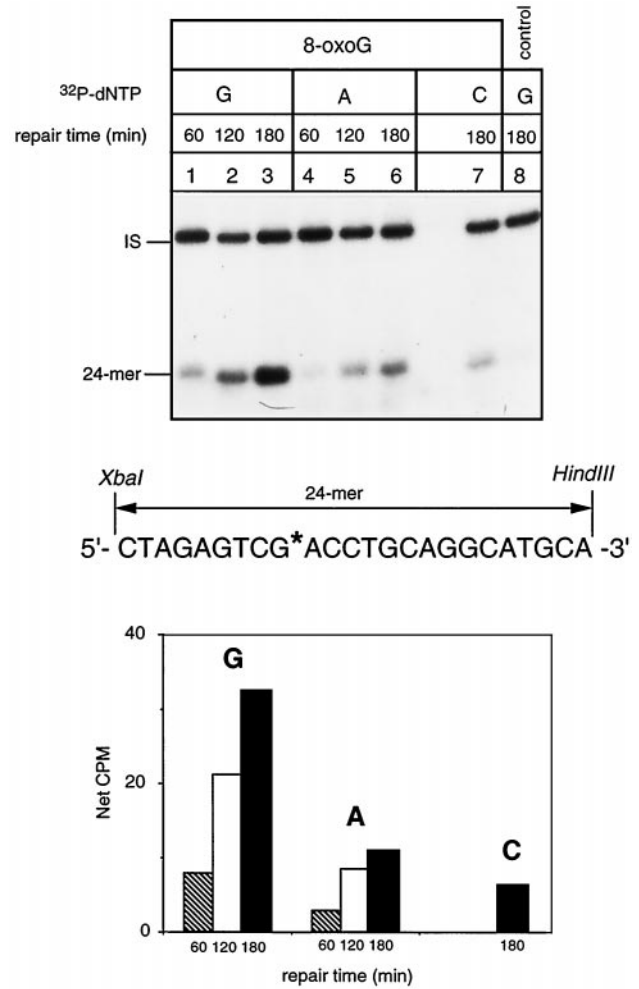
**FIG. 3. Repair of 8-oxoG as compared with a preformed AP site by HeLa cell extracts.** Repair replication was performed for different periods of time in the presence of [ $\alpha$ -<sup>32</sup>P]dGTP, [ $\alpha$ -<sup>32</sup>P]dCTP, or [ $\alpha$ -<sup>32</sup>P]dTTP as indicated. The plasmid DNA was then digested with *Xba*I-*Hind*III to release the 24-bp fragment originally containing the lesion. *A*, autoradiograph of a denaturing polyacrylamide gel. Lanes 1–6, repair synthesis at the 8-oxoG residue. Lanes 1–3, short patch repair synthesis as a function of the incubation time; lanes 4–6, long patch repair synthesis as a function of the incubation time. Lanes 7–12, repair synthesis at the AP site. Lanes 7–9, short patch repair synthesis as a function of the incubation time; lanes 10–12, long patch repair synthesis as a function of the incubation time. Lane 13, control plasmid. Lane 14, following repair replication, the plasmid DNA containing 8-oxoG was digested with *Xba*I-*Hinc*II to release a 8-bp fragment as shown in the scheme. *B*, repair efficiency of 8-oxoG as compared with a preformed AP site. The repair products were measured by electronic autoradiography and relative incorporation, corrected for DNA recovery, is indicated on the ordinate (Net CPM).

components required for BER except PCNA (data not shown) was used to verify whether the repair of 8-oxoG was affected by the presence of PCNA. The CFII fraction (Fig. 4, lanes 4–6) was able to perform the short patch BER of the oxidized base. The addition of PCNA did not modify the extent of DNA repair in the case of short incubation times (lanes 7 and 8), whereas an increase of the full repair product was observed after 2 h of repair (lane 9). This increase is likely to reflect filling in of gaps longer than 1 nucleotide due to strand displacement reactions stimulated by PCNA. Because the repair reaction is largely independent from the presence of PCNA, this is an additional argument suggesting that neither NER nor long patch BER play a significant role in the repair of this lesion.

A fine mapping of the repair patch was performed using different radiolabeled dNTPs in the repair reaction. Fig. 5 shows that the large majority of the nucleotide replacements in the reactions are confined to 1 nucleotide (lanes 1–3), approx-



**FIG. 4. Requirement of PCNA for repair synthesis at 8-oxoG by HeLa cell extracts.** Repair replication was performed for different periods of time in the presence of [ $\alpha$ - $^{32}$ P]dGTP. The plasmid DNA was then digested with *Xba*I-*Hind*III to release the 24-bp fragment originally containing the lesion. *Top*, autoradiograph of a denaturing polyacrylamide gel is shown. *Lanes 1–3*, repair synthesis by whole cell extracts (WCE) as a function of the incubation time; *lanes 4–6*, repair synthesis by the CFII fraction as a function of the incubation time; *lanes 7–9*, repair synthesis by the CFII fraction after addition of PCNA (50 ng) as a function of the incubation time. *Bottom*, the repair products were measured by electronic autoradiography, and relative incorporation, corrected for DNA recovery, is indicated on the ordinate (Net CPM).



**FIG. 5. Characterization of the repair patch at 8-oxoG by HeLa cell extracts.** After repair replication the plasmid DNA was digested with *Xba*I-*Hind*III to release the 24-bp fragment originally containing the lesion. *Top*, autoradiograph of a denaturing polyacrylamide gel. *Lanes 1–3*, repair synthesis in the presence of [ $\alpha$ - $^{32}$ P]dGTP as a function of the incubation time; *lanes 4–6*, repair synthesis in the presence of [ $\alpha$ - $^{32}$ P]dATP as a function of the incubation time; *lane 7*, repair synthesis in the presence of [ $\alpha$ - $^{32}$ P]dCTP after 180 min of incubation time; *lane 8*, control plasmid. *Bottom*, the repair products were measured by electronic autoradiography, and relative incorporation, corrected for DNA recovery, is indicated on the ordinate (Net CPM).

imately one-third of the repair events involve 2 nucleotides (*lanes 4–6*), and very rarely exceed this size including 3–7 nucleotides downstream to the lesion (*lane 7*). As expected, no repair synthesis was detected in the undamaged control plasmid (*lane 8*).

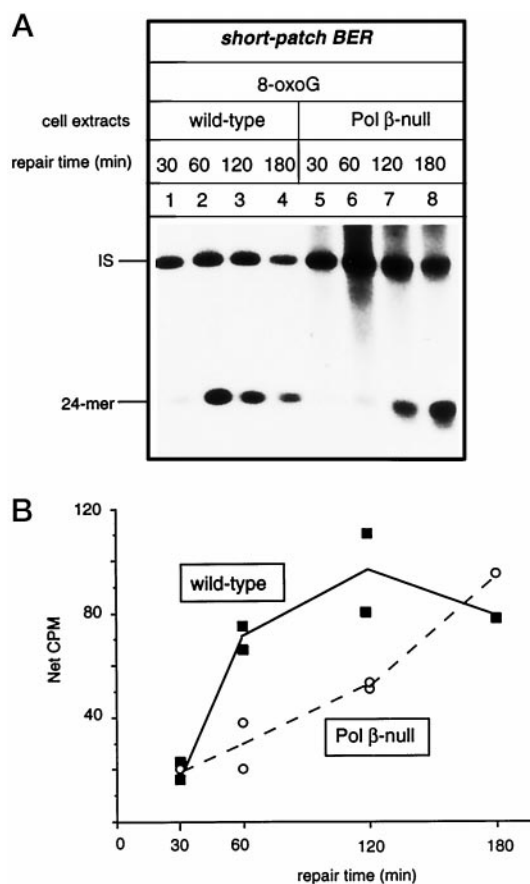
**Pol  $\beta$  Is the DNA Polymerase of Election in 8-oxoG Repair—**Pol  $\beta$  is the major DNA polymerase implicated in the single-nucleotide gap filling synthesis in BER (27, 30, 31). Therefore, extracts from mouse Pol  $\beta$ -knockout cells and their isogenic wild-type cells (20) were tested for their ability to repair 8-oxoG residues. As shown in Fig. 6, we observed a striking difference in the amount of repair when wild-type cell extracts (*lanes 1–4*) were compared with Pol  $\beta$ -defective extracts (*lane 5–8*). In the absence of Pol  $\beta$ , almost no repair was detected after 1 h of repair time (*lane 6*), whereas the repair process using wild-type cells extracts had by that time reached a plateau (Fig. 6B). Moreover, when purified rat Pol  $\beta$  (10 ng) was added to the Pol  $\beta$ -null extract (Fig. 7, *lanes 7 and 8*), the repair activity was restored to the level measured with wild-type extracts (*lanes 1*

and 2). Taken all together these results strongly suggest that Pol  $\beta$  is the polymerase of election for filling in the gap created by the excision of 8-oxoG. Pol  $\beta$ -defective extracts were eventually able to repair the gap after 3 h of incubation (Fig. 6A, *lane 8*), suggesting a possible back-up system that is Pol  $\beta$ -independent.

Mouse cell extracts were tested for their ability to perform long patch BER at 8-oxoG lesions. As shown in Fig. 8, mouse extracts were unable to perform detectable repair synthesis of three or more nucleotides at this lesion (*lanes 1–3*) extending to other species the results reported above for human cells. This type of long patch repair synthesis was only detected with Pol  $\beta$ -defective extracts (*lanes 4–6*), although the extent of activity was at the threshold of detection (*lane 6*).

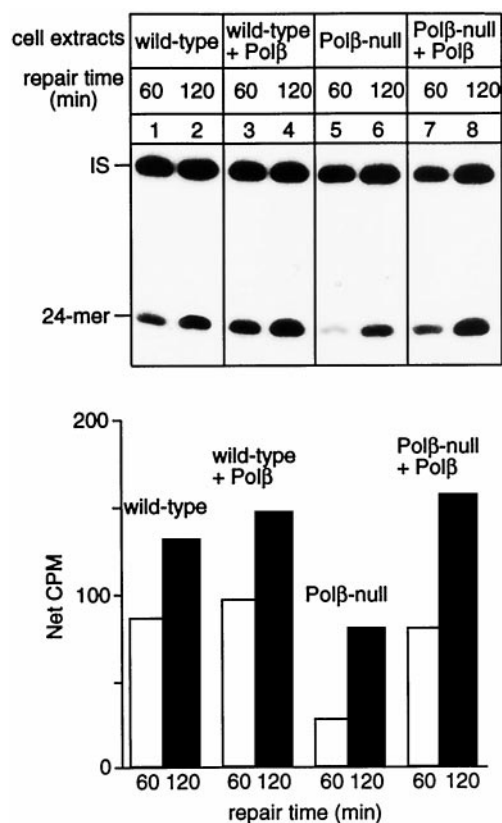
DISCUSSION

In the BER pathway, the initial step is mediated by specific DNA-glycosylases that excise the modified base and thus generate an AP site. There are two classes of DNA glycosylases. An example of the first one is the ANPG protein, the human



**FIG. 6. Repair of 8-oxoG by wild-type and Pol  $\beta$ -deleted mouse cell extracts.** Repair replication was performed for different periods of time in the presence of [ $\alpha$ - $^{32}$ P]dGTP. The plasmid DNA was then digested with *Xba*I-*Hind*III to release the 24-bp fragment originally containing the lesion. *A*, autoradiograph of a denaturing polyacrylamide gel. *Lanes 1–4*, repair synthesis by wild-type cell extracts as a function of the incubation time; *lanes 5–8*, repair synthesis by Pol  $\beta$ -null cell extracts as a function of the incubation time. *B*, the repair products were measured by electronic autoradiography, and relative incorporation, corrected for DNA recovery, is indicated on the ordinate (*Net CPM*).

3-methyladenine DNA glycosylase, a homolog of the *E. coli* Alk A protein, whose only catalytic function is the cleavage of the C1'-*N*-glycosidic bond, liberating the modified base and generating an AP site in DNA. This AP site is believed to be further processed by the HAP1/APEX protein, an AP endonuclease that generates on the 5' side a nick having a 3'-hydroxyl group (reviewed in Ref. 13). An example of the second class of DNA glycosylase is the OGG1 protein, the homolog of the *E. coli* Fpg protein, that excises oxidized purines. Besides its DNA glycosylase activity, this protein is endowed with a AP lyase activity that nicks DNA at AP site by a  $\beta$ -elimination mechanism (28, 32). It generates on the 3' side a nick having a 5'-phosphate. Therefore the products generated by these two classes of glycosylases are different. In mammalian cells, two pathways are involved in AP site repair, the short patch (1-nucleotide gap filling) and the long patch (2–6 nucleotide resynthesis) BER (reviewed in Ref. 33). We hypothesized that structural different modified bases, being recognized by DNA glycosylases having different intrinsic properties, will drive the repair process to either the short or the long patch pathway. In this study we showed that, indeed, HX and  $\epsilon$ A, excised by the monofunctional ANPG protein, are repaired via both the short and long patch BER, whereas 8-oxoG residues, excised by the bifunctional OGG1 protein, are repaired mainly by the short patch



**FIG. 7. Effect of additional Pol  $\beta$  on 8-oxoG repair by wild-type and Pol  $\beta$ -deleted mouse cell extracts.** Repair replication was performed for different periods of time in the presence of [ $\alpha$ - $^{32}$ P]dGTP. The plasmid DNA was then digested with *Xba*I-*Hind*III to release the 24-bp fragment originally containing the lesion. *Top*, autoradiograph of a denaturing polyacrylamide gel. *Lanes 1 and 2*, repair synthesis by wild-type cell extracts as a function of the incubation time; *lanes 3 and 4*, after addition of 10 ng of purified rat Pol  $\beta$ ; *lanes 5 and 6*, repair synthesis by Pol  $\beta$ -null cell extracts as a function of the incubation time; *lanes 7 and 8*, after addition of 10 ng of purified rat Pol  $\beta$ . *Bottom*, the repair products were measured by electronic autoradiography, and relative incorporation, corrected for DNA recovery, is indicated on the ordinate (*Net CPM*).

pathway.

In the case of HX and  $\epsilon$ A, under our experimental conditions, the relative efficiency of the two BER branches was similar to what previously reported for the repair of preformed AP site, suggesting that the initial step, *i.e.* the cleavage by the DNA N-glycosylase, is not rate-limiting. In the case of 8-oxoG, the repair kinetics was much slower than that observed for the repair of preformed abasic site, suggesting that in this case the initial step(s), *i.e.* the cleavage by the glycosylase/AP lyase and/or the 3' terminus-removing activity, could drive the efficiency of the process.

In *E. coli* 8-oxoG residues are repaired by both BER and NER (34). Our data show that in mammalian cells most of 8-oxoG lesions are repaired by the short patch BER in a PCNA-independent mechanism. However, our observations do not preclude a small contribution of NER to repair of oxidative DNA damage (35), which might go undetected in our repair assay.

To attain the sensitivity required for detection of the repair patches we used appropriately purified circular duplex substrates containing modified nucleotides having stable glycosidic bonds located at specific positions of the plasmid genome. The lesions that are introduced by exogenous damaging DNA compounds generate substrates that have serious drawbacks; the specificity is not absolute, and the lesions are randomly

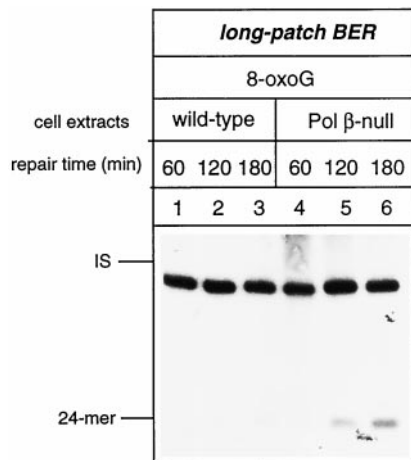


FIG. 8. Long patch repair synthesis at 8-oxoG by wild-type and Pol  $\beta$ -deleted mouse cell extracts. Repair replication was performed for different periods of time in the presence of [ $\alpha$ - $^{32}$ P]dCTP. The plasmid DNA was then digested with *Xba*I-*Hind*III to release the 24-bp fragment originally containing the lesion. Autoradiograph of a denaturing polyacrylamide gel is shown. Lanes 1–3, repair synthesis by wild-type cell extracts as a function of the incubation time; Lanes 4–6, repair synthesis by Pol  $\beta$ -null cell extracts as a function of the incubation time. IS, internal standard.

distributed in the molecule. The use of different types of damaged DNA substrates (single lesion *versus* randomly damaged plasmids) might explain some discrepancies between our findings and those by Jaiswal *et al.* (36) on the repair pathways at 8-oxoG lesions.

Based on our results we propose that the type of DNA glycosylase that initiates the BER process determines which branch of BER is selected to restore the original DNA template. Our findings suggest a model where the formation of a 5' abasic terminus by the sequential action of a monofunctional glycosylase, like ANPG, and of a 5'-AP endonuclease, like HAP1/APE, and its slow processing by a dRPase activity will determine the long patch repair events that occur in competition with the predominant one-gap filling reactions. The status of the 5' terminus would then have a functional role as already suggested by the finding that the dRP lyase activity of Pol  $\beta$  is rate-determining in a reconstituted BER system *in vitro* using AP endonuclease, Pol  $\beta$ , and DNA ligase I (16).

In agreement with this model, the formation of a 3' blocked terminus by the glycosylase/AP-lyase activity of OGG1 is preferentially followed by single nucleotide replacement reactions. In this case the determinant of the repair synthesis step could be the production of 3' OH primers (likely by the 3' phosphoesterase activity of HAP1) because the 5' terminus produced by the AP-lyase is ready for the ligation step.

While this manuscript was under revision, two studies were published on the BER of oxidative DNA damage. Klungland *et al.* (37), by reconstituting *in vitro* the BER of oxidized pyrimidines that are a substrate for the hNth protein, a bifunctional DNA glycosylase (reviewed in Ref. 1), showed that this type of BER is strictly a 1-nucleotide replacement pathway. A model similar to that inferred from our data is proposed implying that the presence of a genuine 5' nucleotide residue minimizes strand displacement events. The second paper by Dianov *et al.* (38) reported that mammalian cell extracts repair 8-oxoG lesions preferentially via single nucleotide replacement reactions (75% of the repair events). In this study, in agreement with the findings reported above, the contribution of NER to the repair of this lesion was not significant.

The limited efficiency of repair of 8-oxoG, also observed by Dianov *et al.* (38), leaves open the question of whether our

experimental conditions are optimal for detecting this repair process. It is not possible to rule out that cofactors that might stimulate, for example, the affinity of the DNA glycosylase for the target lesion are lost or not functional in our assay. Moreover, several reports suggest that the repair of oxidative lesions might be inducible. The major mammalian AP endonuclease, HAP1/APEX, whose 3' phosphoesterase activity removes 3' blocking groups generated in DNA by glycosylase/AP lyase activity, has been shown to be activated specifically by reactive oxygen species (39). This activation is paralleled by an increased cell resistance to the cytotoxicity of reactive oxygen species generating agents. These findings are compatible with the hypothesis that HAP1, via its phosphoesterase activity, is the rate-limiting step in BER of oxidative lesions.

In previous studies (21, 23) we have shown that Pol  $\beta$ -defective cell extracts are able to compensate for the lack of Pol  $\beta$  by using Pol  $\delta$  and/or  $\epsilon$  for repair synthesis following AP site incision. Also in the case of 8-oxoG repair back-up systems seem to be able to substitute for Pol  $\beta$  because Pol  $\beta$ -null cells are eventually able to repair these lesions although at a much slower rate than wild-type cells. The understanding of the biological relevance of these back-up repair systems *in vivo* waits further studies on the genotoxic effects in Pol  $\beta$ -null cells of various BER-inducing agents including reactive oxygen species generators.

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## **The Type of DNA Glycosylase Determines the Base Excision Repair Pathway in Mammalian Cells**

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