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Different DNA Polymerases Are Involved in the Short- and Long-Patch Base Excision Repair in Mammalian Cells†

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ABSTRACT: Mammalian cells possess two distinct pathways for completion of base excision repair (BER): the DNA polymerase β (Pol β)-dependent short-patch pathway (replacement of one nucleotide), which is the main route, and the long-patch pathway (resynthesis of 2−6 nucleotides), which is PCNA-dependent. To address the issue of how these two pathways share their role in BER the ability of Pol β-defective mammalian cell extracts to repair a single abasic site constructed in a circular duplex plasmid molecule was tested in a standard in vitro repair reaction. Pol β-deficient extracts were able to perform both BER pathways. However, in the case of the short-patch BER, the repair kinetics was significantly slower than with Pol β-proficient extracts, while the efficiency of the long-patch synthesis was unaffected by the loss of Pol β. The repair synthesis was fully dependent on PCNA for the replacement of long patches. These data give the first evidence that in cell extracts DNA polymerases other than Pol β are specifically involved in the long-patch BER. These DNA polymerases are also able to perform short-patch BER in the absence of PCNA, although less efficiently than Pol β. These findings lead to a novel model whereby the two BER pathways are characterized by different protein requirements, and a functional redundancy at the level of DNA polymerases provides cells with backup systems.

Spontaneous DNA damage and small lesions induced by a variety of exogeneous agents are corrected by the components of the base excision repair (BER)[1] pathway. The primary pathway for BER involves the action of a DNA glycosylase that generates an AP site that is then cleaved by a 5′-AP endonuclease. A phosphodiesterase excises the 5′ deoxyribose phosphate to leave a single nucleotide gap, which is then filled by a DNA polymerase and sealed by a DNA ligase. This pathway, called short-patch BER, has been reconstituted in vitro with bacterial proteins (*1*) and more recently with purified human proteins (*2*, *3*; Prasad et al., manuscript in preparation). The combined action of human uracil-DNA glycosylase, HAP1, DNA polymerase β, and either DNA ligase III or DNA ligase I has been shown to successfully repair a uracil residue present in a duplex oligonucleotide (*2*). Although this scheme is commonly used by all organisms, it is by no means the only route for the repair of base damage. The AP site can be the target not

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[1] Abbreviations: BER, base excision repair; Pol β, DNA polymerase β; AP, apurinic/apyrimidinic.

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only for a hydrolytic AP endonuclease but also for an AP lyase like the *Escherichia coli* endonuclease III or its recently cloned human homolog, hNTH1 (*4*). Moreover, a gap greater than one nucleotide can be generated at the base damage site, and the completion of repair in this case is PCNA-dependent (*5*, *6*). In vitro reconstitution of this second BER pathway (*7*), called long-patch BER, has confirmed that PCNA is required for this process and has shown that the structure-specific nuclease DNase IV is essential for cleavage of the reaction intermediate produced by template strand displacement. Since PCNA plays an essential role as an auxiliary factor for DNA polymerases δ/ϵ (Pol δ/ϵ) in SV40 replication (*8*) and nucleotide excision repair in vitro (*9*), the requirement of PCNA strongly suggests that DNA polymerases other than Pol β are involved in the long-patch BER synthesis step.

To address the issue of the identity of the DNA polymerases involved in BER and to evaluate the relative contribution of the two pathways to the repair process in vivo, we employed Pol β-deficient cell extracts to assay the repair of a single abasic site present on a circular duplex DNA.

EXPERIMENTAL PROCEDURES

DNA Substrates. Closed circular DNA containing a single uracil residue was produced as previously described (*6*) by priming single-stranded (+) pGEM-3Zf DNA (Promega) with a 30-fold molar excess of uracil-containing or control oligonucleotide and incubating with T4 DNA polymerase holoenzyme, single-stranded DNA binding protein and T4 DNA ligase (Boehringer Mannheim). Closed circular DNA duplex molecules were purified by cesium chloride equilibrium centrifugation. The oligonucleotides 5′GATCCTCTAGAGTCGUCCTGCA3′ and 5′GATCCTCTAGAGUCGACCTGCA3′ were used to create plasmid molecules containing a single uracil residue. These circular closed DNAs were then digested with *E. coli* uracil-DNA glycosylase (a kind gift of S. Boiteux, CNRS/CEA, France) to produce a single abasic site (pGEM-X). The oligonucleotide 5′GATCCTCTAGAGTCGACCTGCA3′ was used to prepare the control plasmid.

DNA Repair Reaction. Nuclear extracts from mouse fibroblasts and whole cell extracts from HeLa cells were prepared as previously described (*10*, *11*). Repair reactions were carried out essentially as described in ref *6*. Briefly, a standard reaction mixture contained 20 μg of extract protein in reaction buffer containing 20 μM each of dNTP and 2 μCi of either [α-^{32}P]dATP, −dCTP, or −dTTP (3000 Ci/mmol) as indicated. After different incubation times at 30 °C, the plasmid DNA was recovered and digested with the appropriate restriction enzymes. The digestion products were resolved on 7% top/20% bottom polyacrylamide gel or on denaturing 15% polyacrylamide gel as indicated. The repair products were visualized by autoradiography and analyzed by electronic autoradiography (Istant Imager, Packard) for quantification.

Antibodies. Anti-PCNA polyclonal antibodies 3009 were a kind gift of L. S. Cox (University of Oxford, Oxford, U.K.). Cell extracts were preincubated for 20 min at 30 °C with 4.6 μg of antibodies before performing the repair reaction (*6*). Neutralizing polyclonal antibodies raised against human Pol β were obtained as previously described (*12*). These antibodies do not cross-react with the other DNA polymerases nor with PCNA (*12*). Cell extracts were preincubated with 50 μg of antibodies for 10 min at 4 °C to inhibit Pol β activity before repair.

RESULTS

Characterization of the Short- and Long-Patch BER. The experimental system used in this study is to assay in vitro the repair ability of mammalian cell extracts by using a circular duplex DNA containing a single lesion as substrate (*13*). The two BER pathways are identified, and their relative efficiency is measured by performing the repair assay in the presence of different radiolabeled nucleotides. In particular, the long-patch BER (i.e., the replacement of 2−6 nucleotides) is specifically measured when the repair reaction is performed in the presence of [α-^{32}P]dCTP (Figures 1 and 2, bottom). Conversely, the short-patch BER (i.e., the replacement of a single nucleotide) is measured by the incorporation of [α-^{32}P]dAMP (in the case of the plasmid containing the U:T base pair, Figure 1, bottom) or [α-^{32}P]dTMP (in the case of the plasmid containing the U:A base pair, Figure 2, bottom) at the lesion site. This value measures mainly the occurrence of one-nucleotide gap filling reactions since the contribution of the PCNA-dependent long-patch BER to the overall repair of abasic sites by human cell extracts is limited to approximately 20% (manuscript in preparation).

The ability of HeLa cell extracts to repair a series of model BER lesions, (i.e., dUMP residue, regular AP site, and AP endonuclease-generated single-strand break) constructed in a circular duplex plasmid DNA was investigated (Figure 1). After repair replication, the double-stranded plasmid was restricted and analyzed by neutral polyacrylamide gel electrophoresis. In every case incorporation of radiolabed nucleotides, either dAMP or dCMP, was specifically associated with the 8-bp digestion product containing originally the lesion (lanes 1−6) but not with the control fragment containing the A:T base pair (lanes 7 and 8). Therefore, in HeLa cell extracts both repair systems, the short- and long-patch BER, concur to the repair of a single uracil residue (lanes 1 and 2) and of its repair intermediates (the abasic site and the HAP1-cleavage product, lanes 3−6) when present in a circular duplex substrate. The intensity of the signal associated with the 8-bp fragment was similar when comparing the repair product obtained with [α-^{32}P]ATP versus [α-^{32}P]dCTP in the reaction mixture (compare lane 1 with 2, 3 with 4, and 5 with 6). This finding confirms that all lesions tested are repaired by both short- and long-patch resynthesis events. A 2−3-fold higher incorporation of radiolabeled dCMP as compared to dAMP is expected if only long-patch (2−6 nucleotides) BER is taking place at the lesion site.

Kinetic experiments (Figure 2) showed that the short-patch BER, monitored in this experiment by [α-^{32}P]dTMP incorporation (lanes 1−3), is a fast process that is almost complete within 20 min while the long-patch BER (lanes 4−6) is a slower process with a low yield of repaired molecules at short repair times but complete repair within 1 h (the repair incorporation does not increase at 3 h incubation time, data not shown). The slower kinetics is compatible with the occurrence of a strand displacement reaction. The repair

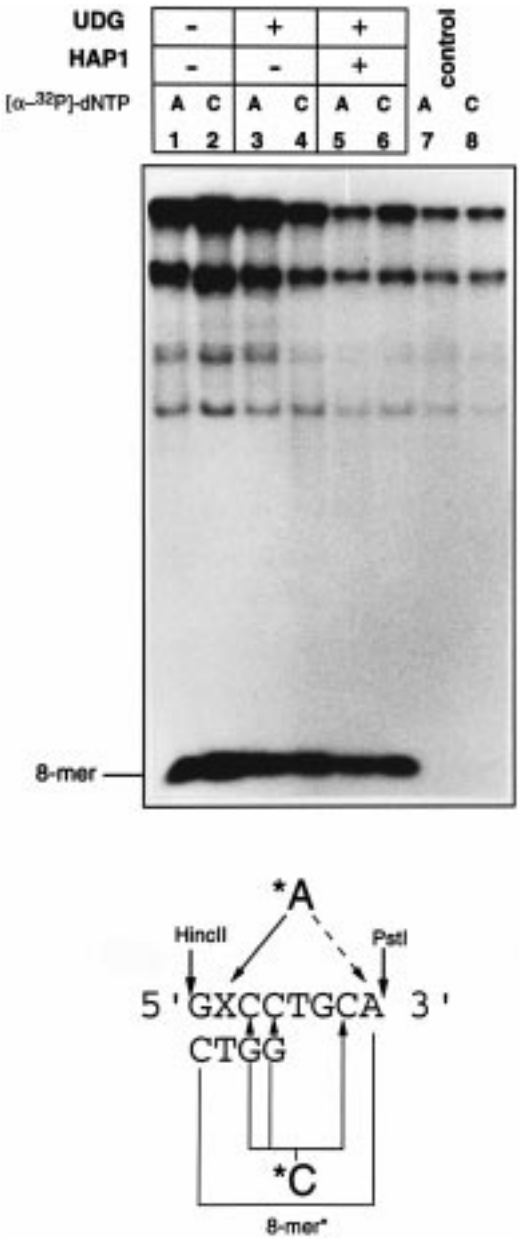

FIGURE 1: Repair of different BER lesions by HeLa cell extracts. (Top) Autoradiograph of a nondenaturing polyacrylamide gel. Repair replication was performed for 1 h in the presence of [α-^{32}P]-dATP or [α-^{32}P]dCTP as indicated. The plasmid DNA was then digested with *Fok*I followed by digestion with *Hinc*II and *Pst*I to release the 8-bp fragment containing originally the lesion. Repair synthesis at a single uracil residue (lanes 1 and 2), abasic site (lanes 3 and 4), and HAP1-induced nick (lanes 5 and 6). The control plasmid (lanes 7 and 8) contains a normal A:T base pair at the lesion site. (Bottom) Scheme of the repair product visualized by autoradiography. The X marks the original position of the lesion. The sites of incorporation of labeled dAMP or dCMP are indicated.

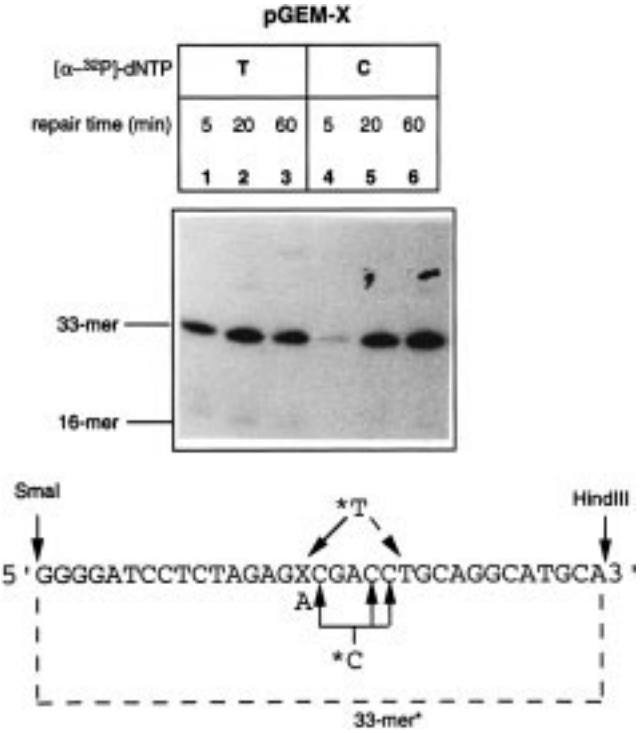

FIGURE 2: Repair of a single abasic site by HeLa cell extracts. (Top) Autoradiograph of a denaturing polyacrylamide gel. Repair replication was performed for 1 h in the presence of [α-^{32}P]dTTP or [α-^{32}P]dCTP as indicated. The plasmid DNA was then digested with *Sma*I and *Hin*dIII to release the 33-bp fragment containing originally the lesion. Unligated products are expected to run with an oligonucleotide ≥16 nt. 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. (Bottom) Scheme of the repair product visualized by autoradiography. The X marks the original position of the AP site. The sites of incorporation of labeled dTMP or dCMP are indicated.

products were visualized in this experiment following denaturing polyacrylamide gel electrophoresis and autoradiography. The DNA *N*-glycosylase (UDG) and the AP endonuclease (HAP1) cleavage reactions are very fast and do not affect the repair rate (data not shown). The rate-limiting step in both repair reactions appears to be the polymerization step. In fact no accumulation of reaction intermediates (≥16 nt oligonucleotides) was observed (Figure 2) even at short incubation times, indicating that the joining was achieved immediately after synthesis. This implies that the release of the 5′-terminal deoxyribose phosphate from the incised AP site (as in the short-patch BER) or that the strand displacement and excision reaction (as in the long-patch BER) precede or are more efficient than the polymerization step. A faint band corresponding to the 16-mer unligated product was occasionally seen in some experiments. The unligated products, when visible, did not decrease as a function of the incubation time, suggesting that they may arise from strand interruptions protected by poly(ADP-ribose)polymerase, which inhibits the final joining step (*14*).

BER in Pol β-Deficient Cell Extracts. Pol β catalyzes DNA synthesis during BER. In fact, cells deleted of both copies of Pol β do not repair a single uracil residue present in a 51-bp duplex substrate (*15*). In contrast, when a circular duplex plasmid containing a single abasic site was incubated for 1 h in the presence of nuclear extracts from embryonic fibroblast cell lines homozygous for Pol β deletion (Figure 3), both short- and long-patch repair synthesis occurred at the lesion site. Extracts from Pol β-deleted cells were able to perform repair synthesis involving one (lane 3) or more (lane 4) nucleotides as efficiently as nuclear extracts from wild-type (lanes 1 and 2) and Pol β-deleted cell lines expressing a mammalian Pol β-minigene (lanes 5 and 6) when [α-^{32}P]dATP and [α-^{32}P]dCTP were used to monitor short- and long-patch repair incorporation, respectively. In this experiment, the repair products were analyzed by neutral

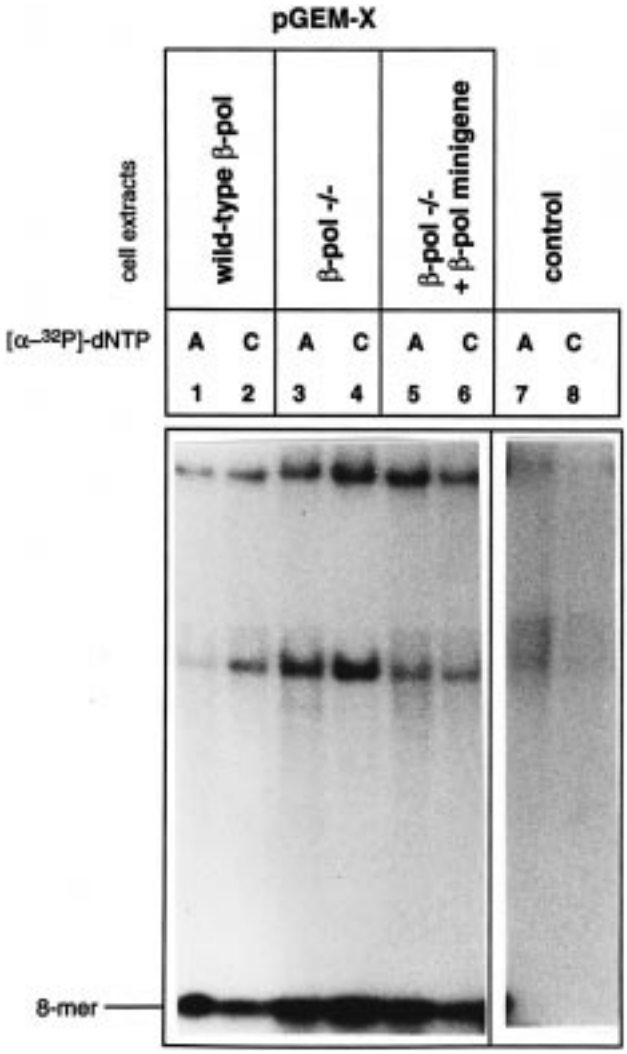

FIGURE 3: Repair of a single abasic site by Pol β-deleted mouse fibroblasts extracts. Autoradiograph of a nondenaturing polyacrylamide gel is shown. Repair replication was performed for 1 h in the presence of [α-^{32}P]dATP or [α-^{32}P]dCTP as indicated. The plasmid DNA was then digested with *Fok*I followed by digestion with *Hin*cII and *Pst*I to release the 8-bp fragment containing originally the lesion (see Figure 1). Repair synthesis by wild-type (lanes 1 and 2), Pol β-deleted (lanes 3 and 4), and Pol β-deleted expressing a Pol β-minigene (lanes 5 and 6) nuclear extracts. The control plasmid (lanes 7 and 8) contains a normal A:T base pair at the lesion site.

polyacrylamide gel electrophoresis. As expected, no repair was detected in the 8-bp fragment containing the control T:A base pair following incubation with Pol β-null cell extracts (lanes 7 and 8).

In a previous study (*6*), we showed that the short-patch BER does not require PCNA while the long-patch BER is PCNA-dependent. The same repair reactions were run after preincubation of nuclear extracts with the 3009 PCNA-neutralizing antibody and analyzed by denaturing gel electrophoresis. As shown in Figure 4, the response of repair incorporation to PCNA inhibition was very similar in the presence (lanes 1−4) or in the absence of Pol β (lanes 5−8). In both cell systems, PCNA is absolutely required for the long-gap filling process (marked by [α-^{32}P]dCMP incorporation) (lanes 4 and 8) while [α-^{32}P]dTMP incorporation seems to be mostly PCNA-independent (lanes 2 and 6). This feature confirms that short-patch repair events occur also in the absence of Pol β.

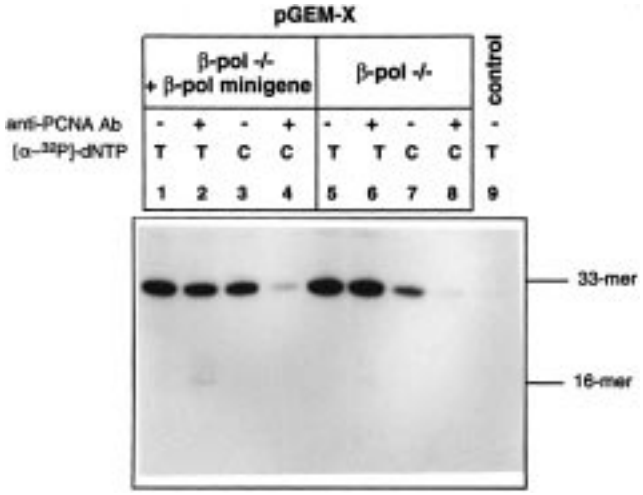

FIGURE 4: PCNA dependence of the short- and long-patch BER performed by Pol β-deleted mouse fibroblasts extracts. Autoradiograph of a denaturing polyacrylamide gel is shown. Repair replication was performed for 1 h in the presence of [α-^{32}P]dTTP or [α-^{32}P]dCTP as indicated. The plasmid DNA was then digested with *Sma*I and *Hin*dIII to release the 33-bp fragment containing originally the lesion. Unligated products are expected to run with an oligonucleotide $\geq$16 nt. Lanes 1 and 3: repair synthesis by Pol β-deleted + Pol β-minigene nuclear extracts; lanes 2 and 4: after preincubation of extracts with anti-PCNA antibody; lanes 5 and 7: repair synthesis by Pol β-deleted nuclear extracts; lanes 6 and 8: after preincubation of extracts with anti-PCNA antibody. The control plasmid (lane 9) contains a normal T:A base pair at the lesion site.

Altogether these data show that, besides Pol β, other polymerases can perform the BER synthesis step. In these Pol β-independent BER systems, PCNA is not required for one-gap filling reactions, while it is indispensable for long-patch repair synthesis.

Although the redundancy in the BER system has been already well documented in other organisms (*16*), our finding that Pol β could be replaced by other polymerases in the major route for AP site repair (i.e., the short-patch repair) was in conflict with the strong evidence of its key role in BER. In fact Pol β seems to be designed for one-gap filling reactions for its dual role in the DNA polymerization step and in the release of the 5′ terminal deoxyribose phosphate residues from incised abasic sites (*17*). Moreover, it has been recently shown that Pol β interacts with XRCC1 (*2*), which is present in a heterodimer with DNA ligase III and has been shown to form a complex with DNA ligase I (*18*). These findings are compatible with the idea of a very efficient BER repair complex that assembles at the AP site and via Pol β favors one-nucleotide gap filling reactions. In agreement with these biochemical data, Pol β-deficient cells are hypersensitive to the cytotoxic effect of monofunctional alkylating agents (*15*). Therefore, we decided to explore the efficiency of short-patch BER as a function of time in Pol β-depleted extracts. As shown in Figure 5, the fast repair component of wild-type cells (almost 50% repair in 10 min, lane 1) was strongly reduced in the defective cells (lane 5), but repair signals similar to those observed with wild-type extracts were observed at later repair times (compare lane 2 with lane 6). Interestingly, a slight inhibition of the repair synthesis at both repair times was observed with the defective extracts in the presence of PCNA neutralizing antibody (lanes 7 and 8), while the repair efficiency of wild-type extracts

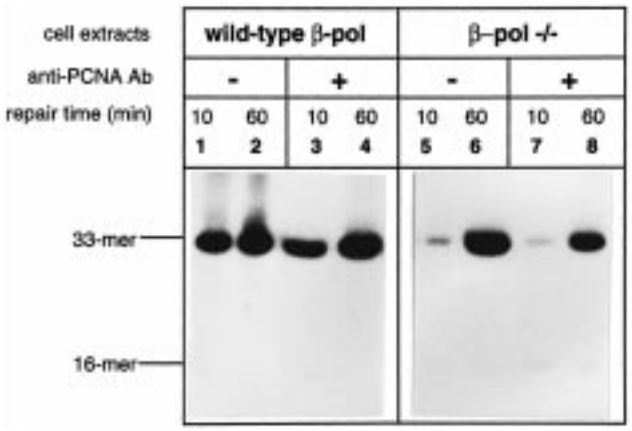

FIGURE 5: Short-patch repair kinetics in Pol β-deleted mouse fibroblasts extracts. Autoradiograph of a denaturing polyacrylamide gel is shown. Repair replication was performed for 10 and 60 min in the presence of [α-^{32}P]dTTP. The plasmid DNA was then digested with *Sma*I and *Hin*dIII to release the 33-bp fragment containing originally the lesion. Unligated products are expected to run with an oligonucleotide $\geq$16 nt. Lanes 1 and 2: repair synthesis by wild-type extracts as a function of time; lanes 3−4: after preincubation of extracts with anti-PCNA antibody; lanes 5 and 6: repair synthesis by Pol β-deleted extracts as a function of time; lanes 7−8: after preincubation of extracts with anti-PCNA antibody.

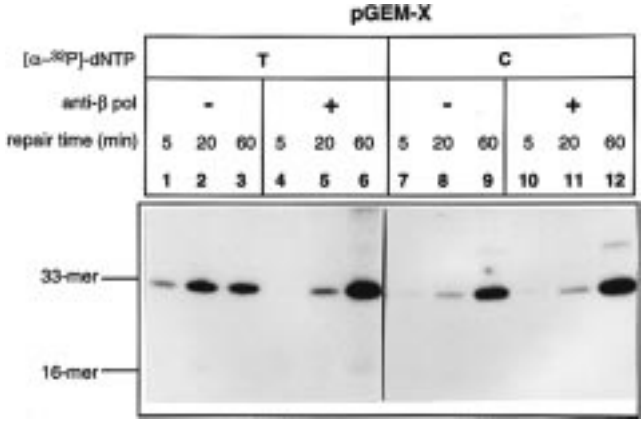

FIGURE 6: Pol β dependence of the short- and long-patch BER performed by HeLa cell extracts. Autoradiograph of a denaturing polyacrylamide gel is shown. Repair replication was performed for 5−60 min in the presence of [α-^{32}P]dTTP or [α-^{32}P]dCTP as indicated. The plasmid DNA was then digested with *Sma*I and *Hin*dIII to release the 33-bp fragment containing originally the lesion. Unligated products are expected to run with an oligonucleotide $\geq$16 nt. Lanes 1−3: short-patch repair synthesis as a function of time; lanes 4−6: after preincubation of extracts with anti-Pol β antibody; lanes 7−9: long-patch repair synthesis; lanes 10−12: after preincubation of extracts with anti-Pol β antibody.

was unaffected by the absence of PCNA (lanes 3 and 4). The long-patch BER kinetics were unaffected by the defect in Pol β (data not shown).

These findings indicate that the defect in Pol β leads to a delay in short-gap filling reactions while it leaves unaltered the PCNA-dependent repair pathway. This delay might reflect the slow processing of 5′-deoxyribose phosphate moieties in the absence of the β-elimination activity of Pol β. The persistence of unprocessed 5′ abasic termini might favor long-patch resynthesis events over the one-nucleotide replacement reactions. This phenomenon might account for the decrease in repair synthesis observed in the absence of PCNA with Pol β-defective cell extracts.

To ascertain whether the existence of Pol β-independent BER systems was confined to rodent cell extracts, the efficiency of BER as a function of time was explored in HeLa cell extracts in the presence of Pol β-specific antibodies. As shown in Figure 6, repair occurred also in the presence of Pol β neutralizing antibodies via both short-patch (lanes 1−6) and long-patch (lanes 7−12) resynthesis. Again, in the case of short-patch BER the fast repair component was significantly reduced in the Pol β-depleted cells as compared to wild-type cells (compare lane 1 with lane 4). In the case of long-patch BER, no significant difference in repair rate was recorded between wild-type and depleted extracts (compare lanes 7−9 with lanes 10−12).

These data confirm that in a wild-type background Pol β is likely to play a major role in the processing of abasic sites via one gap-filling reactions, while DNA polymerases other than Pol β would be the main "players" in the long-patch BER events.

DISCUSSION

This work presents the first evidence that DNA polymerases other than Pol β are involved in the long-patch BER in mammalian cell extracts and can act as backup polymerases in the short-patch BER when Pol β is defective. We have shown that repair of uracil and regular AP sites by human cell extracts is achieved by both short- and long-patch repair synthesis. In the absence of Pol β, both repair pathways occurred. However, while the rate of long-patch resynthesis was unaffected, a delay in the short-gap filling reactions as compared to Pol β-proficient extracts was observed.

The present in vitro data lead to a new model for BER in mammalian cells. DNA abasic sites present in a circular duplex plasmid are repaired via both short- and long-patch repair synthesis. The short-patch BER is the predominat route and is efficiently performed by the sequential and likely coordinated action of HAP1, Pol β, and DNA ligase I or III (*12*, *2*, *18*, *3*). This pathway presents a functional redundancy at the level of DNA polymerase. The repair rate is slower in the absence of Pol β, thus confirming that Pol β is the polymerase of election for one-nucleotide gap filling reactions.The long-patch BER, which is a minor route of repair of abasic sites (but might be the preferential route for other lesions), involves the action of different components. PCNA is absolutely required to achieve efficient repair and, under these conditions, Pol δ/ϵ can efficiently perform the synthesis step. Whether PCNA is required for its interaction with the DNA polymerase or with the flap endonuclease FEN1 that might be involved in the cleavage of the resulting overhang (*7*) remains to be established. A preference for DNA ligase I in the sealing step is suggested (*19*).

The model recently proposed for the repair of abasic sites in mammalian cells is based on data obtained by using a modified double-stranded oligonucleotide as substrate (*7*). Uracil residues as well as regular AP sites when present in this linear substrate were shown to be repaired by human cell extracts by one-nucleotide gap filling reactions. Longer repair patches were only observed with a reduced AP site. Pol β antibodies strongly suppressed this repair reaction although the reaction could be reconstituted with either Pol β or Pol δ. We believe that the discrepancy with our data

is mainly due to the different DNA substrate, linear versus covalently closed circular duplex DNA, used to monitor DNA repair. The efficiency of assembly of repair/replication complexes as well as the efficiency of DNA synthesis might be seriously affected by the geometry of the DNA substrate (*20*). In our study, the use of a circular duplex substrate identified BER components whose role might be hidden by the constraints imposed by a linear substrate for protein complex assembly and function. It is interesting to note that the new findings of this study involve a potential role for DNA polymerases that are stimulated by or dependent on PCNA for their activity. It is well established that a circular structure of the DNA substrate is absolutely required for auxiliary proteins, like the RFC/PCNA complex, to interact with DNA polymerases (*21*, *20*). Therefore, the DNA substrates used in several studies (*22*, *2*, *7*) might be poor templates for PCNA-dependent repair reactions.

The functional redundancy in the BER process is not unique to mammalian cells. In yeast cells, evidence has been presented for the involvement of three DNA polymerases in the BER process: Pol δ (*23*), Pol ϵ (*16*), and Pol β (*24*). As a possible consequence of this redundancy, the disruption of the polymerase IV gene, which encodes for the Pol β homologue in *Saccharomyces cerevisiae* (*25*), results either in no phenotype (*26*) or in only slight sensitivity to methyl methanesulfonate (MMS) (*27*).

In light of our discovery of the existence of Pol β-independent BER systems, alternative explanations for the phenotype of Pol β-knockout mouse cells (*15*) can be envisaged. The hypersensitivity of Pol β-deleted mammalian cells to the killing effects of monofunctional alkylating agents, like MMS, might be explained by the longer persistence in the cell genome of single-strand breaks (due to a slower resynthesis and/or ligase step by thePol β-independent BER system) more than by a complete inability to perform BER. The lack of phenotype reported when hydrogen peroxide or ionizing radiation were used as DNA-damaging agents would also be explained since the induction of oxidized AP sites might push the BER process toward the long-patch pathway, which is Pol β-independent. In fact, there are already data indicating that structurally distinct lesions are repaired in a different manner by the BER system. The Pol β-dependent BER is unable to repair reduced AP sites as well as tetrahydrofuran residues (*5*), and repair patches of 2−6 nucleotides in length were found after repair of reduced or oxidized AP sites (*7*). In this scenario where two BER systems evolved to handle structurally different lesions, there are also interesting indications that some BER (we think the long-patch BER) components might interact with proteins required for transcription-coupled repair (*28*). The PCNA-dependent BER pathway might be closer to the NER system than we have ever thought.

NOTE ADDED IN PROOF

Biade et al. have recently shown that the PCNA-dependent AP site repair is functional on circular but not on linear DNA in vitro (*29*).

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