Overexpression of DNA polymerase β results in an increased rate of frameshift mutations during base excision repair

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DNA polymerase β (Pol β) is important for the base excision repair (BER) pathway. Overexpression of Pol β is frequently found in cancer cells and is thought to be associated with tumorigenesis. In this study, we examined BER fidelity in extracts derived from a human lymphoblastoid cell line that overexpresses Pol β compared to normal control cells. Using an in vitro mutagenesis assay, we found an increased rate of frameshift mutations arising during DNA repair in whole-cell extracts derived from the Pol β-overexpressing cells. We demonstrate that the addition of excess Pol β to a control cell extract enhances the mutagenic potential of the extract. Furthermore, using cell extracts and purified Pol β, we demonstrate that the mechanism of frameshift formation involves slippage of Pol β during the one-nucleotide gap-filling step of BER and that this slippage is fixed by strand-displacement synthesis stimulated by an excess of Pol β.

Introduction

Base excision repair (BER) is a highly co-ordinated chain of enzymatic reactions that is responsible for the identification, removal and replacement of aberrant DNA bases (1). The majority of BER is accomplished through a short-patch repair pathway where only one nucleotide (1-nt) is removed and replaced. Damage-specific DNA glycosylases recognize and remove the aberrant base via cleavage of the N-glycosyl bond, creating an abasic site that is incised on the 5’-side by apurinic/apyrimidinic endonuclease 1 (APE1), leaving a 3’-hydroxyl group and a 5’-deoxyribose phosphate group flanking the nucleotide gap. DNA polymerase β (Pol β) then inserts 1-nt into the gap and at the same time removes the 5’-deoxyribose phosphate residue. Finally, the DNA ligase IIIα-X-ray repair cross-complementing protein-1 (XRCC1) heterodimer seals the DNA ends (2). DNA repair synthesis is a vulnerable step in BER since mistakes made during this process can give rise to mutations. Consequently, the fidelity of BER is mainly dependent on the ability of Pol β to incorporate the correct nucleotide into a repair gap. Pol β does not have any 3’-5’ exonuclease proofreading activity and, when filling long DNA gaps, Pol β is known to be error prone (3). However, filling of 1-nt gaps by Pol β is reasonably accurate, although not completely error free (4). Surprisingly, when the nature of mutations arising during repair of DNA base lesions in mammalian cell extracts was analysed, the majority of them were found to be 1-nt deletions (3,5,6). Interestingly, it was also demonstrated that 1-nt deletions are characteristic for both short-patch and long-patch (replacement of 2–8 nt) BER pathways (7). Pol β participates in both these subpathways of BER by adding the first nucleotide into the repair gap (8) and it is plausible that the same mechanism, involving Pol β, may be responsible for generating 1-nt deletions in both pathways. It was suggested previously that a ‘DNA repair synthesis misalignment’ mechanism is involved in the formation of 1-nt deletions during BER (7). According to this model, Pol β skips a nucleotide in the repair gap and instead incorporates the appropriate nucleotide downstream of the gap. Further incorporation of additional nucleotides then stabilizes the misalignment and fixes the 1-nt deletion. According to this model, an excess of DNA polymerase may lead to an enhanced level of strand-displacement synthesis, and this would increase stability of misaligned structures and may result in increased rate of deletion formation. Indeed, it was found that a 2- to 4-fold increased level of Pol β in mammalian cells can increase the mutation rate (9); however, the nature of the mutations and the mechanism involved have not been identified.

In this study, we addressed whether the frequency of 1-nt deletions generated during BER is increased in human cell extracts overexpressing Pol β, and also investigated the ability of recombinant Pol β to generate 1-nt deletions during BER reconstituted with purified enzymes.

Materials and methods

Materials

Synthetic oligodeoxyribonucleotides, purified by high-performance liquid chromatography, were obtained from MWG-Biotech (Ebersberg, Germany) and further purified by gel electrophoresis. [γ-32P]ATP (6000 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were purchased from Amersham Biosciences (Little Chalfont, UK) and BDH Laboratory Supplies (Poole, UK), respectively. QIAprep Spin M13 Kit and BigDye Terminator (v3.1) Cycle Sequencing Kit were purchased from Qiagen (Crawley, UK) and Applied Biosystems (Foster City, CA), respectively.

Proteins

Recombinant human uracil-DNA glycosylase (UDG) was purified as described previously (10). Histidine-tagged Pol β and APE1 were purified on Ni-NTA agarose (Qiagen) as recommended by the manufacturer. XhoI restriction endonuclease was purchased from Promega. T4 DNA ligase, T4 DNA polymerase and T4 gene 32 protein were purchased from Roche. T4 polynucleotide kinase was from NEB.

Antibodies

Proliferating cell nuclear antigen antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against rat Pol β was raised in rabbit and affinity purified as described (11).
Cells and extracts
The Ha cell line was obtained from the Cancer Research UK Cell Services, and derived from a 53-year-old man with a stage I, high grade, B-cell lymphoma located at the back of the throat. During the first phase of radiotherapy (15 fractions of 2 Gy per fraction), he exhibited a severe oral reaction necessitating the introduction of nasogastric feeding. Six months later, spinal cord damage was evident which was succeeded by apical pulmonary fibrosis. The GP cell line was isolated from a patient with leukaemia whose radiotherapy needed to be stopped due to an adverse reaction. The Jb cell line was isolated from a normal, healthy, male volunteer and RZM was isolated from a normal female volunteer. Neither of the normal volunteers had a history of cancer or radiation sensitivity. All cell lines were transformed with Epstein-Barr virus in the Cancer Research UK Cell Services Laboratory, Clare Hall, UK. Cells were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum, 1% l-glutamine and 1% of penicillin and streptomycin. Whole-cell extracts (WCEs) were prepared by the method of Tanaka et al. (12), as modified by Vodenicharov et al. (13), and were dialysed overnight against buffer containing 25 mM HEPES–KOH (pH 7.9), 0.1 M KCl, 12 mM MgCl₂, 17% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). Extracts were aliquoted and stored at –80°C.

Western blots
Western blots were performed by a standard procedure as recommended by the vendor (Novex, San Diego, CA). Blots were visualized using the ECL plus detection system (Amersham Biosciences).

DNA substrates
Closed-circular, double-stranded, M13 DNA substrates containing a 1 nt gap were prepared as follows. Single-stranded M13mp18 DNA was isolated from Escherichia coli JM109 and purified according to Kunkel et al. (14). A site-specific uracil was introduced into the lacZa gene sequence of a double-stranded closed-circular M13mp18 DNA using a 5'-phosphorylated 33 mer oligonucleotide (5'-pGGCTGCAGGTGCTGACATUTAGAGATCCCGGTTA-3') as previously described (15). The 1 nt gap containing DNA substrate was freshly prepared on the day of the experiment by incubating 200 ng of uracil-containing substrate with 30 ng of human UDG for 20 min at 37°C in 40 mM HEPES–KOH (pH 7.8) containing 5 mM MgCl₂, 0.1 mM EDTA and 0.5 mM dithiothreitol (DTT). Humanized nanograms of human APE1 and 50 ng of human Pol β were added, and further incubated for 20 min at 37°C. DNA was then phenol-chloroform extracted and filtered through a Sepharose G-25 spin column (Amersham Biosciences) equilibrated with 10 mM Tris–HCl (pH 8.0). To prepare the 1 nt gap-containing oligonucleotide substrates, the upstream primer (5'-GCCTGCAGGTGCTGACATUTAGAGATCCCGGTTA-3', 30 pmol) was 5'-end labelled with 20 μCi (33 pmol) of [γ-32P]ATP using T4 polynucleotide kinase. The labelled upstream primer was mixed with downstream primer (5'-TAGAGATT-CACGGGT-3', 30 pmol) and the template primer (5'-ACCGTGATTTCC-TAGTAGCTGACCTGAGCC-3', 60 pmol), and the mixture was incubated at 90°C for 5 min, and then slowly cooled to room temperature in annealing buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 0.1 M NaCl.

In vitro gap-filling reactions
Repair reactions utilizing 32P-labelled 1 nt gap oligonucleotide substrates (6 ng, 300 fmol) were reconstituted in a reaction mixture (10 μl) containing 45 mM HEPES–KOH (pH 7.8), 70 mM KCl, 7.5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT, 0.5 mg/ml bovine serum albumin (BSA) and 20 μM each of the indicated dNTPs, unless otherwise stated. The reactions were initiated by the addition of purified Pol β at the concentration indicated in the figure legends. After incubation for 20 min at 37°C, the reactions were stopped by the addition of 10 μl gel-loading buffer (95% formamide, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol). Following incubation at 95°C for 5 min, the reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel.

In vitro BER reactions with WCE
Repair reactions of 32P-labelled 1 nt gap oligonucleotide substrates (6 ng, 300 fmol) were carried out in a reaction mixture (20 μl) containing 50 mM HEPES–KOH (pH 7.8), 50 mM KCl, 10 mM MgCl₂, 0.5 mM EDTA, 1.5 mM DTT, 2 mM ATP, 0.1 mg/ml BSA, 25 mM phosphorothioate (di-Tris salt, Sigma), 2.5 μg creatine phosphatase (type I, Sigma), 8.5% glycerol, 20 μM each of the dNTPs, unless otherwise stated, and 400 ng carrier DNA (100 ng single-stranded oligonucleotide). Reactions were initiated by the addition of WCE (4 μg), incubated for 20 min at 37°C and were stopped by the addition of 20 μl of gel-loading buffer. Following incubation at 95°C for 3 min, the reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel.

M13 BER reactions with WCE
Repair reactions of 1 nt gap closed-circular M13 DNA substrates (100 ng) with WCEs were carried out in 50 μl of reaction mixture as described previously (7).

Reactions were initiated by the addition of 100 μg of WCE, or WCE extract supplemented with 40 ng Pol β, then incubated for 30 min at 37°C and were stopped by the addition of 2 μl 0.5 M EDTA, 2 μl 10% sodium dodecyl sulphate (SDS) and 2 μl Proteinase K (5 mg/ml). After incubation for a further 30 min at 37°C, the substrate DNA was purified from the reaction mixture by phenol–chloroform extraction and filtered through a Sepharose G-25 spin column equilibrated with 10 mM Tris–HCl (pH 8.0). To select ‘mutant DNA’, in vitro-repaired DNA was treated overnight at 37°C with or without 40–60 units of XhoI restriction endonuclease and used for bacterial cell transformation.

Transformation of E. coli and determination of mutation frequency
An equal amount (1 ng) of restriction endonuclease digested or undigested in vitro-repaired DNA was introduced into E. coli XL-1 Blue by the calcium chloride method (16). The transformed cells were diluted in SOC medium and were plated on LB agar containing 0.2 mM of IPTG and 40 mg/ml of X-gal. Plates were incubated at 37°C for 16 h. The mutation frequency was calculated as the ratio of the number of colourless plaques obtained from digested DNA to the total number of plaques obtained from undigested DNA. Colourless plaques were picked, transferred into 1 ml of LB and were stored at 4°C overnight. The extracted phages were diluted in LB media and titrated against E. coli XL-1 Blue on LB agar plates containing IPTG and X-gal. An individual colourless plaque after secondary screening was used to isolate phage DNA for nucleotide sequence analysis.

Determination of mutation spectrum
Colourless plaques obtained from the secondary screening were used to isolate M13mp18-derived single-stranded DNA according to the manufacturer’s protocol (QIAprep Spin M13 Kit, Qiagen). The DNA sequence was determined on an ABI 3100 Genetic Analyser using sequencing primer M13mp18-200 (Applied Biosystems).

Results
In order to further our understanding of the biological consequences of a misbalance in BER enzymes, and, in particular, the effect of Pol β overexpression on DNA repair fidelity, we performed a search for cells naturally overexpressing Pol β. When the levels of BER enzymes were compared in cells derived from a healthy person (Jb) to lymphoblastoid cell lines derived from cancer patients who exhibited a radiation hyper reaction, we identified one cell line (Ha) with an ~6-fold-overexpressed levels of Pol β (Figure 1A). We did not find any changes in the levels of other BER enzymes tested including APE1, XRCC1 (data not shown) and PCNA (Figure 1A). Moreover, Ha cells were shown to overexpress Pol β compared to other normal human cell lines.

Cells overexpressing Pol β were shown previously to have increased rates of mutagenesis, although the nature of these mutations and the mechanisms involved were not identified (9). To analyse this, we measured the frequency of mutations in wild-type (Jb) and the Pol β-overexpressing Ha cells using an in vitro mutagenesis assay developed in our laboratory (7). In addition, we analysed the fidelity of DNA repair synthesis in these cells. For the in vitro mutagenesis assay, we constructed a closed-circular, double-stranded, M13mp18 DNA substrate containing a site-specific uracil residue within the lacZa gene (Figure 1B, steps I & II). The uracil was further converted into a 1 nt gap by treatment with UDG, APE1 and Pol β in the absence of dNTPs (Figure 1B, step III). The substrate DNA was then incubated with WCE derived from Jb or Ha cells (Figure 1B, step IV), purified, treated with XhoI restriction endonuclease to select mutants and was used for transformation into bacterial cells (Figure 1B, steps V & VI). Colourless mutant plaques were selected on X-gal-containing plates and the phage DNA was sequenced.

In the first series of experiments, we investigated the frequency of mutations generated during repair of a substrate
containing a 1-nt gap by WCEs. We observed an \( \frac{9}{3} \)-fold increase in mutation frequency in the Pol\(b\) overexpressing Ha extracts compared to the wild-type Jb extracts (Table I). To investigate whether the increased frequency of mutations was due to the elevated level of Pol\(b\), we performed repair reactions in Jb WCE supplemented with 40 ng per reaction of recombinant human Pol\(b\). We found that this addition of excess Pol\(b\) to Jb cell extracts increased the mutation frequency by \( \frac{6}{3} \)-fold, compared to unsupplemented Jb extract alone (Table I). To characterize the mutations, DNA from selected colourless plaques was purified and sequenced. Mutations located within this region from nucleotide \(-15\) (5'-upstream to the 1-nt gap) to nucleotide +50 (3'-downstream to the 1-nt gap, direction of DNA repair synthesis) were analysed. We found that in all three WCE (Ha, Jb and Jb + Pol\(b\)), 40–60% of mutations were located within the repair gap (Table II) and nearly all of them were single-nucleotide deletions (Figure 2). We concluded that the elevated level of Pol\(b\) is responsible for the increased mutation rate in Ha WCE and that a majority of the induced mutations are arising as a result of Pol\(b\)-generated single-nucleotide frameshifts.

We proposed previously a mechanism of single-nucleotide frameshift mutation formation that involves two major components: slippage of Pol\(b\) and stabilization of the frameshift by further strand-displacement synthesis (7). To elucidate the mechanism of increased mutagenesis in cells overexpressing Pol\(b\), we examined whether more strand displacement occurs in Ha WCE during a gap-filling reaction compared to wild-type extracts. First, using a 1-nt gap oligonucleotide substrate, we conducted DNA repair synthesis reactions reconstituted with purified Pol\(b\) and demonstrated that the efficiency of strand-displacement synthesis was proportional to the amount of the enzyme used (Figure 3A). Similarly, we observed \( 1.64 \pm 0.06 \)-fold more

### Table I. Frequency of mutations produced by BER in human WCE. A 1-nt gap containing substrate was repaired in human WCE generated from wild-type cells (Jb), Pol\(b\)-overexpressing cells (Ha), or Jb extract supplemented with 40 ng Pol\(b\). After repair, DNA was purified and transformed into bacterial cells. The mutation frequency was calculated as described in Materials and Methods, and the results of three independent experiments are shown with the calculated mean and standard deviation.

<table>
<thead>
<tr>
<th>Cell extract</th>
<th>Mutation frequency/10^4</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jb</td>
<td>7.14</td>
<td>9.14</td>
<td>3.08</td>
<td>6.5 ± 3.12</td>
<td></td>
</tr>
<tr>
<td>Ha</td>
<td>50.79</td>
<td>61.35</td>
<td>63.49</td>
<td>58.5 ± 6.80</td>
<td></td>
</tr>
<tr>
<td>Jb + Pol(b)</td>
<td>37.67</td>
<td>47.62</td>
<td>25.97</td>
<td>37.1 ± 10.83</td>
<td></td>
</tr>
</tbody>
</table>

### Table II. Distribution of different types of mutations generated during repair of 1-nt gap containing M13mp18 DNA in human WCE. DNA from randomly selected mutants generated during repair by WCE from wild-type cells (Jb), Pol\(b\)-overexpressing cells (Ha), or Jb extract supplemented with 40 ng Pol\(b\) were purified and sequenced. Relative amounts of specific mutations are shown with percentages in brackets.

<table>
<thead>
<tr>
<th>Mutation type</th>
<th>Jb</th>
<th>Ha</th>
<th>Jb + Pol(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-base deletions in the repair gap</td>
<td>14 (41.2)</td>
<td>32 (65.3)</td>
<td>16 (53.3)</td>
</tr>
<tr>
<td>Other single-base deletions</td>
<td>3 (8.8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mutations outside (-15/-50)</td>
<td>17 (50.0)</td>
<td>17 (34.7)</td>
<td>14 (46.7)</td>
</tr>
<tr>
<td>Total number sequenced</td>
<td>34</td>
<td>49</td>
<td>30</td>
</tr>
</tbody>
</table>
strand-displacement products generated during repair in Ha WCE-overexpressing Pol β in comparison with wild-type Jb WCE (Figure 3B).

Finally, to demonstrate that Pol β is able to generate a 1-nt frameshift during the gap-filling reaction, we carried out DNA synthesis reactions in the presence of deoxythymidine triphosphate (TTP) and deoxyadenosine triphosphate (dATP) only. Since the first four nucleotides on the 5′-side downstream of the gap are GATC (Figure 4A), there are only three possible outcomes of the repair synthesis in the presence of TTP and dATP: (i) misincorporation of TMP opposite to G, followed by TMP and dAMP incorporation resulting in an extension of three nucleotides (Figure 4A, product I); (ii) misincorporation of dAMP opposite to G, followed by TMP and dAMP incorporation, also resulting in an extension of three nucleotides (Figure 4A, product II) and (iii) template slippage of Pol β and incorporation of TMP opposite to A, followed by dAMP incorporation, resulting in an extension of two nucleotides (Figure 4A, product III).

Using purified Pol β, even at low concentrations of dNTPs, we observed a small proportion of two nucleotide incorporation products that become more prominent at high TTP concentrations. This suggests that, under these conditions, slippage of Pol β dominates over misincorporation opposite to G (Figure 4B). To demonstrate that product III can be further extended to generate a frameshift structure (Figure 4A, product IV), we carried out gap-filling reactions in the presence of three dNTPs (TTP, dATP and dGTP). Pol β was able to efficiently extend the two nucleotide slippage products, thus fixing a frameshift mutation (Figure 4C).

Discussion

Pol β plays a central role in BER. Pol β variants that affect the control of incoming nucleotide selection may be a major source of mutations during repair. Direct sequencing of the pol β gene from different cancer cells has identified a number of mutations. To date, 189 tumours of various subtypes have been characterized, and 54 (30%) of these were shown to express variants of the Pol β protein. Among these variants, single amino acid alterations were the most prevalent (17). Sweasy and colleagues have extensively studied the fidelity of DNA synthesis associated with colon and prostate cancer-associated Pol β variants. They found that cancer-associated Pol β gene mutations give rise to polymerase with a strong mutator activity (18). Moreover, expression of at least some of these tumour variants in mouse cells leads to cellular transformation (19).

Much less is known about the mechanism of instability induced by Pol β overexpression. Previous identification of Pol β overexpression in some cancer cells raised the question of the possible role of misregulated Pol β expression on genome stability and tumourigenesis (20,21). Recently, Albertella and colleagues used expression arrays to systematically study the expression patterns of Pol β in cancer cells (22). They found that Pol β was overexpressed at the mRNA and protein level in approximately one-third of all tumours sampled and was most frequently overexpressed in cancers of the uterus, ovary, prostate and stomach (22). Hoffmann’s group engineered cell lines to overexpress Pol β and demonstrated an increased mutation frequency in these
Pol β overexpression and mutagenesis

Fig. 4. Pol β induced frameshift formation during repair of 1-nt gap containing DNA. (A) Models for 1-nt gap-filling reactions and formation of frameshift mutations. See text. Asterisks denote the 5’-32P radiolabel. (B) A 1-nt gap containing oligonucleotide substrate (300 fmol) was incubated with 200 fmol of purified Pol β for 20 min at 37°C in a buffer containing the indicated amount of TTP and dATP. Reaction products were analysed by electrophoresis on a 20% denaturing polyacrylamide gel and by phosphorimaging. (C) Lane 1, 1-nt ladder marker. Lane 2, no dNTPs control. Lane 3, 800 μM TTP and 100 μM dATP. Lane 4, 800 μM TTP, 100 μM dATP and 100 μM dGTP.

cells (9). Moreover, by injecting immunodeficient nude mice with various control and Pol β-overexpressing cells, it was shown that cells expressing an excess of Pol β can induce cancer. Less than 25% of control injections induced the formation of small, slow-growing tumours, whereas >70% of Pol β-overexpressing injections induced rapidly growing carcinomas (23). These experiments suggest a direct link between Pol β overexpression and elevated levels of mutations, and support the ‘mutator phenotype’ hypothesis of tumorigenesis proposed by Loeb et al. (24). However, the molecular mechanism underlying the mutator phenotype of Pol β-overexpressing cells has remained obscure.

Pol β is the key DNA polymerase involved in BER and it was recently shown that 1-nt frameshifts are the major class of mutations arising during this process (3,5–7). The mechanism suggested for frameshift formation relies on template slippage of DNA polymerase and stabilization of the frameshift by further strand-displacement synthesis (7). Because Pol β is a distributive enzyme, it dissociates from DNA substrates after addition of the first nucleotide into a repair gap. We propose that addition of a second nucleotide (and, as a result, stabilization of misalignment) will require repetition of the Pol β association–dissociation cycle. Consequently, the probability of stabilizing misalignments by addition of a second nucleotide into a repair gap, as well as the probability of frameshift mutation formation, will be higher if the Pol β concentration is elevated. In this study, we tested this model experimentally. We selected a cell line overexpressing Pol β and demonstrated increased strand-displacement synthesis and mutation rate during BER in cell extracts derived from these cells. We then analysed the mutation spectrum and found that the majority of mutations are 1-nt frameshifts. The simplest explanation for the single-nucleotide deletion would be “over-gap” ligation of the DNA ends without filling the gap. However, we have previously demonstrated that 1-nt frameshifts are specific to gap-filling step of BER since they are also specific for repair of reduced AP sites, that cannot be repaired by direct ligation (7). Moreover, addition of excess Pol β to the wild-type cell extract resulted in a 6-fold increase in the frequency of 1-nt frameshifts. Finally, using purified Pol β, we experimentally demonstrated template slippage of Pol β during gap filling and slippage stabilization by further strand displacement. Taken together, these data strongly support the proposed model for mutagenesis via Pol β during BER and suggest that overexpression of Pol β in vivo may result in genomic instability.

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References


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