Long-patch DNA repair synthesis during base excision repair in mammalian cells

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The base excision repair (BER) process removes base damage such as oxidation, alkylation or abasic sites. Two BER sub-pathways have been characterized using *in vitro* methods, and have been classified according to the length of the repair patch as either 'short-patch' BER (one nucleotide) or 'long-patch' BER (LP-BER; more than one nucleotide). To investigate the occurrence of LP-BER *in vivo*, we developed an assay using a plasmid containing a single modified base in the transcribed strand of the enhanced green fluorescent protein (EGFP) gene and a stop codon, based on a single-nucleotide mismatch, at varying distances on the 3' side of the lesion. The reversion of the stop codon occurs after DNA repair synthesis and restores EGFP expression after transfection of mismatch-repair-deficient cells. Repair patches longer than one nucleotide were observed for 55–80% or 80–100% of the plasmids with a mean length of 2–6 or 6–12 nucleotides for 8-oxo-7,8-dihydroguanine or a synthetic abasic site, respectively. These data show the existence of LP-BER *in vivo*, and emphasize the effect of the type of BER substrate lesion on both the yield and the extent of the LP-BER sub-pathway.

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INTRODUCTION

Among DNA repair processes, base excision repair (BER) is one of the pathways by which eukaryotic cells correct a variety of DNA damage (Hoeijmakers, 2001). BER is initiated by DNA glycosylases, which are often specific for a particular type of base damage or, more commonly, a group of related types (Scharer & Jiricny, 2001). These enzymes remove the damaged base, leaving a non-instructive apurinic/apyrimidinic (AP) site with mutagenic potential.

The AP site is subsequently processed by at least two BER sub-pathways: 'short-patch' BER (SP-BER) and 'long-patch' BER (LP-BER). SP-BER (one nucleotide) has been reconstituted *in vitro* using four purified human proteins (Kubota *et al.*, 1996). After incision into the sugar–phosphate DNA backbone by an AP

endonuclease, the resulting 5'-blocking desoxyribose residue is removed by a 5' deoxyribophosphatase (dRPase) activity. The principal dRPase activity in mammalian cells is that of DNA polymerase- β (Pol- β) (Srivastava *et al.*, 1998). LP-BER occurs by the excision of at least 2 nucleotides, and often 6–13 nucleotides, and DNA synthesis can be catalysed by Pol- β or by Pol- δ/ϵ (Fortini *et al.*, 1998; Klungland & Lindahl, 1997; Pascucci *et al.*, 1999). LP-BER is dependent on the proliferating cell nuclear antigen (PCNA), and a reconstituted enzyme system has been developed for assaying LP-BER of a reduced AP site using purified human Ap endonuclease 1, Pol- β or Pol- δ , PCNA, Flap endonuclease 1 and Ligase 1 (Klungland & Lindahl, 1997; Matsumoto *et al.*, 1999). The interplay between DNA polymerases in BER sub-pathways is still under discussion (Dogliotti *et al.*, 2001).

Whereas the BER mechanism has been investigated intensively *in vitro*, using cell-free extracts or purified proteins, little information is available *in vivo*. A series of assays monitoring the strand-specific removal of different oxidative base lesions has been developed, and these assays have been used to demonstrate an increased rate of repair of the transcribed strand compared with the non-transcribed strand. This specificity is similar to that observed for nucleotide excision repair (NER), and has been referred to as transcription-coupled repair (TCR; Leadon & Cooper, 1993). However, more precise analysis of BER in the genomic context is hampered by the inability to introduce a single lesion at a defined position on chromosomal DNA. Therefore, investigators have developed systems using plasmids, usually carrying one chemically defined lesion, to probe these pathways. Recently, the *in vivo*, differential removal, from either the transcribed or the non-transcribed strand, of a single 8-oxo-7,8-dihydroguanine (8-oxoG) residue (placed in a shuttle vector) was unambiguously demonstrated, providing direct evidence for TCR of oxidative DNA damage (Le Page *et al.*, 1998; 2000b). Shuttle vectors are now used widely in eukaryotic cells to monitor such diverse processes as repair, mutagenesis and translesion synthesis (Avkin *et al.*, 2002; Brooks *et al.*, 2000).

Several *in vitro* studies examining the preferential use of polymerases in BER, as well as a recent analysis of kinetic parameters, have suggested a predominant role for SP-BER, which questions the relevance of LP-BER (Dianov *et al.*, 1992; Sokhansanj *et al.*, 2002). As direct evaluation of the two BER sub-pathways in the nucleus is difficult, we have developed an indirect approach,

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Fig. 1 | Strategy for determining repair-patch length. (A) Schematic representation of the assay principle. (B) Illustration of the composite substrates used in this study. Both a single lesion (marked X, position +1) and a stop codon, at varying distances from the lesion, were used in the composite substrates, which were based on the coding sequence of the enhanced green fluorescent protein (EGFP). NT, non-transcribed strand. T, transcribed strand. The stars indicate sites of damage.

using the transfection of cells with plasmid DNA containing defined base damage. Our aim was to evaluate the contribution of the two BER sub-pathways, and to estimate the size of the repair patches. Here, we report direct evidence that, following the excision of 8-oxoG or incision at an AP site, a significant proportion of the damage is processed by LP-BER *in vivo*.

Fig. 2 | Analysis of DNA synthesis patches during 8-oxo-7,8-dihydroguanine repair *in vivo*. HCT116 cells were transfected with undamaged (Un), 8-oxo-7,8-dihydroguanine-modified (GO), mismatch-modified (M(+2), M(+6), M(+12) and M(−9)) or composite substrates. The mean fluorescence levels were expressed as percentages of enhanced green fluorescent protein (EGFP) expression from the control (GO) construct. The results shown are mean values from three independent experiments ± s.d.

RESULTS

Assay principle

To test whether LP-BER (more than one nucleotide) occurs *in vivo*, we made a plasmid construct containing a single 8-oxoG residue in the transcribed strand of an enhanced green fluorescent protein (EGFP) reporter gene close to a stop codon that was positioned at various locations on the 3' side of the lesion (Fig. 1A). The stop codon was introduced using an oligonucleotide that created a mismatch in the plasmid DNA. After cell transfection with a closed-circular construct carrying one mismatch, the expression of EGFP should be decreased or abolished. As the mismatch was placed on the 3' side of the base damage, after cell transfection with a composite substrate carrying both the mismatch and the base damage, the expression of EGFP should be restored if the mismatch is corrected during the replication step. Conversely, in the absence of base-damage repair, or when base damage is repaired by the SP-BER sub-pathway, the mismatch should not be corrected. The correction depends on the occurrence of LP-BER, and changing the distance between the lesion and the stop codon in the transcribed strand should permit the evaluation of the size of the repair patch during BER.

Mismatch-dependent inhibition of reporter gene expression

Three stop codons were put into the amino-terminal cassette of the transcribed strand of the EGFP gene, using primer M(+2) to introduce a C/C mismatch and primers M(+6) and M(+12) to introduce a T/T mismatch (Fig. 1B). Each modified substrate was transfected into an MutL homologue 1 mutant cell line (HCT116), which is a mismatch repair (MMR)-deficient cell line. The EGFP background fluorescence in HCT116 transfected cells was decreased to ~20% when either UAG or UAA stop codons (M(+2) or M(+6)) were present, compared with the normal codon (undamaged) that was used

Fig. 3 | Analysis of DNA synthesis patches during repair of a synthetic apurinic/ apyrimidinic site in vivo. HCT116 cells were transfected with tetrahydrofuran-containing substrates (apurinic/apyrimidinic, AP), mismatch-modified substrates (M(+2), M(+6), M(+12)) or composite substrates. The mean fluorescence levels were expressed as percentages of the enhanced green fluorescent protein (EGFP) expression from the control (AP) construct. The results shown are mean values from three independent experiments $\pm$ s.d.

as a control for EGFP expression (Fig. 2). Transfection of HCT116 cells that were reverted to MMR proficiency gave similar results with the C/C mismatch (20% of the signal) but not with the T/T mismatch (28–35%) (data not shown). Consequently, to minimize the background EGFP expression level, HCT116 cells were chosen for subsequent transfection experiments with the different constructs.

8-Oxoguanine is processed by SP-BER and LP-BER in cells

As a model lesion, 8-oxoG repair was tested. No difference in reporter gene expression was found using the substrate containing a single 8-oxoG lesion compared with the control substrate containing neither damage nor mismatch (Fig. 2). As shown in Fig. 2, transfection of the 8-oxoG modified (GO) M(+2) substrate (GO:M(+2)) resulted in an increase in EGFP expression from 20% to 70–80%. In addition, EGFP expression decreased gradually as the distance between the 8-oxoG and the mismatch increased. This gradient is consistent with the occurrence of repair patches of different sizes, with a significant proportion of repaired substrates having a patch longer than six nucleotides. Although the existence of a gradient did not support the possibility of a repair event through the NER pathway, we tested the possibility that a NER reaction was functioning in the removal of 8-oxoG by moving the mismatch to position –9 at the 5' side of the damage (Fig. 1B). As shown in Fig. 2, no significant increase in EGFP expression was obtained from the GO:M(–9) substrate compared with background expression from the control M(+6) construct, confirming that BER alone was responsible for the removal of 8-oxoG.

LP-BER is the main pathway for repair of synthetic AP sites

In the case of 8-oxoG, it was possible that the restoration of protein expression was dependent on non-specific 5' $\rightarrow$ 3' exonuclease activities functioning after strand cleavage. To rule this out, we investigated the repair of a synthetic AP site, a type of damage that is known to be corrected only by LP-BER *in vitro* (Klungland & Lindahl, 1997). EGFP expression with a single modified AP site in the plasmid was identical to that obtained using an unmodified substrate (data not shown).

Fig. 4 | Green fluorescent protein expression with different composite substrates. Expression levels are shown for 8-oxo-7,8-dihydroguanine (8-oxoG) or apurinic/apyrimidinic (AP) sites in composite constructs; results are normalized for the background green fluorescent protein expression associated with the mismatch alone (C/C or T/T) at the corresponding position (expression factor = 1).

Using the composite substrate, we observed full expression of EGFP at position +2 (Fig. 3). The expression decreased as the distance between the AP site and the mismatch increased, but the fluorescence value was still ~50% for the +12 nucleotide substrate.

A comparison between AP site and 8-oxoG repair patch size is shown in Fig. 4. Both the relative mean size of the LP-BER patch and its quantitative contribution to repair in comparison with SP-BER were different for the two types of damage. The mean patch length is 2–6 nucleotides for the 8-oxoG damage and 6–12 nucleotides for the synthetic AP site. Consequently, the lesion, and not the mismatch downstream, predominantly determined the signal that was measured as the release of inhibition of EGFP expression.

DISCUSSION

From *in vitro* assays using either cell extracts or purified proteins, two DNA-repair replication pathways have been shown to be involved in the BER process: 'short-patch' (one nucleotide) and 'long-patch' (more than one nucleotide) DNA repair synthesis. Data from the *in vivo* DNA repair assay that we have developed provide evidence for the existence of long-patch repair synthesis *in vivo*. There is no sequence bias, as the same results were obtained with composite substrates in another sequence context (sequence +295, +302; mismatch position +2, +4, +7; data not shown). The release of EGFP expression inhibition is likely to be linked to repair synthesis after excision of DNA damage for several reasons: first, at a given position, the level of mismatch reversion is different for the two types of damage studied (Fig. 4), and the global profile obtained for each repair synthesis patch is consistent with the corresponding extent of replication expected from *in vitro* data (see below); second, the levels of reversion of 3' mismatches downstream of the lesion were considerably higher than for 5' mismatches (Fig. 2, and data not shown).

The TCR pathway has been shown to function on oxidative lesions, such as thymine glycol and 8-oxoG (Cooper *et al.*, 1997; Le Page *et al.*, 2000b). For the TCR of 8-oxoG, it has been suggested that both the glycosylase-initiated BER and NER pathways function on the transcribed strand (Le Page *et al.*, 2000a). On the basis of *in vitro* estimates, the

action of the NER pathway on the damage in the composite substrate used here would have replaced mismatches upstream of the lesion from positions –22 to –25 (Huang *et al.*, 1992). As no significant reversion of the stop codon was observed at position –9 upstream from the lesion, we conclude that, under our experimental conditions, NER is not operating in the repair pathway.

Because the closest mismatch in our constructs was one nucleotide to the 3' side of the damage, only the contribution of the LP-BER sub-pathway was evaluated. As no difference was seen in EGFP expression from an undamaged control substrate or from a single-site 8-oxoG-damaged substrate (Fig. 2, and data not shown), the kinetics of repair are negligible in the time interval between cell transfection and measurement of EGFP expression. Consequently, for the composite lesion/mismatch (+2) substrates used here, it can be postulated that, for some repair events, the level of EGFP expression is closely related to the number of events associated with repair synthesis longer than one nucleotide.

However, EGFP expression from a single-mismatch construct reaches ~20% of the levels seen for the control substrate. This background expression could be due to: first, an MMR-independent repair mechanism (Fleck *et al.*, 1999) or a transcription or translation bypass, or second, a replication-independent process, possibly operating on residual nicks in a small fraction of the transfected plasmids, and identical for control, unique-site damaged or composite substrates.

Thus, we can infer from our data that, for 8-oxoG lesions and for a synthetic AP site, repair patches are longer than one nucleotide for ~55–80% and ~80–100% of repair events, respectively. Our estimate for LP-BER is higher than that obtained from most *in vitro* assays (Dianov *et al.*, 1998; Fortini *et al.*, 1999; Pascucci *et al.*, 2002), although another study reported that one-nucleotide repair patches account for only 20% of repair events (Bennett *et al.*, 2001). We believe that the proportion of repair synthesis events at the +2 position was overestimated, as a mismatch close to the lesion could impair ligation after a single nucleotide substitution event. Nevertheless, a release of EGFP inhibition still occurred at the +6 position for 8-oxoG and for the AP site, accounting for a significant number of repair patches reaching at least this position (32–53% and 75–96%, respectively). Thus, in the case of 8-oxoG, 32% of the repair events are longer than six nucleotides and 68% are shorter. Alternatively, the participation of LP-BER might be generally underestimated from *in vitro* studies.

In conclusion, this assay should have a wide application for probing DNA repair synthesis in mammalian cells, in testing, for example, other BER substrate lesions or the balance between SP-BER and LP-BER in different *in vivo* situations.

SPECULATION

With regard to the mean length of the repair patches, the difference between the two lesions analysed in this study supports the model in which the lesion governs the selection of the repair synthesis pathway (Fortini *et al.*, 1999). In this respect, it is important to note that repair of two DNA base lesions on opposite strands by LP-BER can result in the formation of cytotoxic DNA double-stranded breaks, as has been shown *in vitro* (Vispe & Satoh, 2000). Thus, our data provide *in vivo* support for the hypothesis that BER, by the mechanism of LP-BER, may contribute to the formation of double-stranded breaks.

METHODS

Plasmid construction and oligonucleotides. The template construct for the assay was based on reporter gene expression from the pGL3–Promoter phagemid (Promega). First, we replaced the SV40 promoter with the cytomegalovirus promoter from pEGFPLuc (Clontech). Second, we inserted the EGFP protein sequence from pEGFPLuc (without ATG) between the cassette and the luciferase gene. Third, a customized sequence was inserted upstream of the EGFP reporter gene, allowing us to study the repair of a single-base modification. The cassette formed by the annealed complementary oligonucleotides (5'-AGCTCGGGATCCTTAGACTTATACCTAGGA-3') (+ strand) and (5'-AGCTTCCTAGGTATAAGTCTAAGGATCCCG-3') (– strand) was inserted into the pGL3–Promoter vector at position +36. The resulting construct, pCMV–kEGFPluc, gives rise to the expression of an N-terminal-modified EGFP–luciferase fusion protein of 809 amino acids. Modified oligonucleotides (30 nucleotides) were made using an automated DNA synthesizer (PerSeptive Biosystems). The phosphoramidite derivative, 8-oxo-dG-CE was incorporated during synthesis. Then, 8-oxo-dG-containing oligonucleotides were purified by electrophoresis on a 10% polyacrylamide/urea gel followed by elution overnight (in a buffer consisting of 0.5 M ammonium acetate, pH 5.0, 10 mM Mg-acetate, 2.5 mM EDTA) and recovery using Sep-Pak C18 Cartridges (Waters Corporation).

Preparation of single-site-modified templates. Phage particles were collected from the supernatant of infected bacterial culture, and the single-stranded circular phage DNA was extracted using phenol/chloroform and precipitated using ethanol. Phage DNA (25 µg) was annealed in 50 µl of buffer with a fivefold molar excess of oligonucleotides, which had been phosphorylated previously using T4 polynucleotide kinase (New England Biolabs). T7 DNA polymerase (100 units) and 1,600 units of T4 DNA ligase (New England Biolabs) were then added, and the polymerization–ligation reaction was carried out at 37 °C for 2 h in a reaction mixture of 200 µl, containing 20 mM Tris/HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 50 µg ml^{–1} BSA, 2 mM ATP and 600 µM dNTPs. Closed circular DNA was purified by caesium chloride density centrifugation.

Cell culture, transfections and analysis of EGFP expression. The HCT116 cell line was cultured in DMEM (Biomedica) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (125 units ml^{–1}), and streptomycin (125 µg ml^{–1}). For transfection experiments, 3 × 10⁵ cells (in 2 ml) were seeded into six-well multidishes and allowed to attach for 1 h. Closed circular double-stranded lesion-containing or lesion-free (as a control) pCMV–kEGFPluc plasmids (1 µg) were transfected into cells using the cationic liposome *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate (DOTAP; Roche) method. After transfection, cells were incubated for 18 h, washed with PBS and recovered by scraping in PBS. The mean fluorescence of 7,000 cells was quantified by flow cytometry (FACScan; Becton Dickinson). The level of fluorescence obtained with undamaged plasmid was about 350–500 arbitrary units. To normalize the fluorescence levels from different experiments, the results were expressed as the ratio of the fluorescence values obtained with damaged versus undamaged plasmids. The efficiency of transfection varied in different experiments between 5% and 7%.

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