Base excision repair intermediates are mutagenic in mammalian cells

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ABSTRACT

Base excision repair (BER) is the main pathway for repair of DNA damage in mammalian cells. This pathway leads to the formation of DNA repair intermediates which, if still unsolved, cause cell lethality and mutagenesis. To characterize mutations induced by BER intermediates in mammalian cells, an SV-40 derived shuttle vector was constructed carrying a site-specific lesion within the recognition sequence of a restriction endonuclease. The mutation spectra of abasic (AP) sites, 5’-deoxyribose-5-phosphate (5’dRp) and 3’-[2,3-didehydro-2,3-dideoxy-ribose] (3’ddR5p) single-strand breaks (ssb) in mammalian cells was analysed by RFLP/PCR and mutation frequency was estimated by quantitative PCR. Point mutations were the predominant events occurring at all BER intermediates. The AP site-induced mutation spectrum supports evidence for the ‘A-rule’ and is also consistent with the use of the 5’ neighbouring base to instruct nucleotide incorporation (5’-rule). Preferential adenine insertion was also observed after in vivo replication of 5’dRp or 3’ddR5p ssb. We provide original evidence that not only the abasic site but also its derivatives ‘faceless’ BER intermediates are mutagenic, with a similar mutation frequency, in mammalian cells. Our findings support the hypothesis that unattended BER intermediates could be a constant threat for genome integrity as well as a spontaneous source of mutations.

INTRODUCTION

The cellular genome stability is constantly threatened by the exposure to endogenous and exogenous agents: alkylating agents, reactive oxygen species and UV-light alter the DNA primary structure. The majority of DNA lesions lead to immediate adverse consequences, such as cell lethality and mutagenesis. In order to limit the deleterious effect of DNA damage, cells are provided of numerous DNA repair mechanisms, including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), recombination and translesion DNA synthesis (TLS). BER is the major repair mechanism involved in the removal of structurally non-distorting and non-bulky lesions, such as oxidized and alkylated bases, deaminated bases and apurinic/apyrimidinic (AP) sites (1). BER is a multi-enzymatic pathway initiated by DNA glycosylases that are able to remove the modified base by cleavage of the N-glycosydic bond giving rise to an AP site. The hydrolytic cleavage by monofunctional DNA glycosylases leads to release of the damaged base and the formation of an AP site, subsequently converted to a single-strand break (ssb) by an AP endonuclease. This activity hydrolyses the phosphodiester DNA backbone 5’ to the AP site leading to 3’-hydroxyl (3’OH) and 5’-deoxyribose-5-phosphate (5’dRp) termini. The removal of the 5’dRp residue, which is essential for the ligation reaction, is catalysed by the 5’dRp lyase activity of DNA polymerase (pol) β. Bifunctional DNA glycosylases present an associated lyase activity that catalyses the cleavage of the DNA phosphodiester backbone via a β-elimination reaction leaving 3’-[2,3-didehydro-2,3-dideoxy-ribose] (3’ddR5p) and 5’ phosphate termini (2). The 3’ddR5p terminus, briefly referred as 3’-αβ-unsaturated aldehyde, is removed by the 3’ phosphodiesterase activity of AP endonuclease 1 (APE 1) to generate a suitable substrate for ligation. The BER reaction can be completed by either short patch (SP-BER) or long patch (LP-BER) repair synthesis. In the SP-BER, the single nucleotide gap is filled by pol β, and X-ray repair cross complementing group I (XRCC I)/LIG III restores DNA strand integrity. Replicative DNA pol δ and ε can replace pol β in the LP-BER. DNA pol δ and ε displace the 5’dRp-containing strand and synthesize longer repair patches. Additional players, FEN I, PCNA, RFC and DNA ligase I, are implicated in this pathway. Reduced or oxidized abasic sites, known to be resistant to the dRp-lyase activity of pol β, are mainly processed by LP-BER (3).

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In the last years, biochemical studies have shown numerous protein–protein interactions among SP- and LP-BER enzymes that led to consider this repair mechanism as an orchestrated process. This might be a requisite to avoid the exposure of highly reactive repair intermediates (4).

The co-ordination among BER components is exemplified by the role of XRCC1, a scaffold protein able to interact with all the enzymes involved in BER following the glycosylase step, such as APE1, DNA pol β and LIG III. XRCC1 could be recruited by the lesion specific glycosylase and then recall the downstream players to repair intermediates (5).

Another example is poly(ADP–ribose) polymerase-1 (PARP 1). Photoaffinity studies showed that PARP 1 is a member of a multi-enzymatic complex which binds to BER intermediates resistant to β-elimination reactions, namely 5′-incised tetrahydrofuran (THF) abasic sites (6). After 5′ssb interaction, PARP 1 seems to stimulate strand displacement synthesis by pol β and flap cleavage by FEN I implying a role of this protein in LP-BER reactions. The biological relevance of a highly co-ordinated BER process is also testitified by recent data that suggest that persistence of ‘unattended’ BER intermediates in the genome is potentially harmful for the cell. Mouse myeloid progenitor bone marrow cells deficient for N-alkylpurine-DNA-glycosylase (7) show increased resistance to alklylation damage, and mouse fibroblasts defective in the lyase activity of DNA pol β are hypersensitive to the cytotoxic and mutagenic effects of alklylation damage (8).

Differnet machanisms are expected to have a role in AP site-induced mutagenesis. In Escherichia coli cells, it has been shown that not only the SOS-dependent TLS is responsible for mutagenesis of chromosomal AP sites, but BER and recombination also play a role (9). In mammalian cells, the crucial role of BER to limit the mutagenic potential of the abasic sites is clearly testitified by the increased spontaneous and alklylation damage-induced mutation frequency observed in pol β-defective cells (10). BER synthesis per se gives a minor contribution to mutagenesis (11,12). In vitro studies on the bypass ability of purified eukaryotic Y-family DNA polymerases strongly suggest the involvement of TLS in BER site-induced mutagenesis in mammalian cells too. However, the relative contribution of TLS in the in vivo mutagenesis of AP sites and the identification of the involved DNA polymerase remain to be elucidated. Finally, stalled DNA replication forks at unrepaird AP sites and non-canonical ssb (i.e. 5′dRp and 3′ddR5p) could be a signal for recombination events. Recently, homologous recombination at double-strand breaks (dsb) arising from unrepaird ssb has been evoked to protect mammalian cells from BER-intermediates-induced cytotoxic effect (13,14).

The mutational specificity of the base loss sites in living cells has been extensively investigated. In bacteria, these lesions become mutagenic under the SOS response and a preferential insertion of an adenine was observed (‘A-rule’). Conversely, in Saccharomyces cerevisiae the tendency for the insertion of dCMP opposite the AP site was found (‘C-rule’) (15).

In mammalian systems, controversial reports either suggest no specificity in the insertion of dNTPs opposite the base loss site or the ‘A-rule’ signature (16,17). No data are available on the mutagenic specificity of other BER repair intermediates, such as the 5′dRp and 3′ddR5p termini. More recently, the mutational specificity of AP sites has been reanalysed following the discovery of a new family of DNA polymerases (Y-family) that successfully replicate damaged DNA templates, such as pol η, pol κ, and Rev1 (18).

Numerous in vitro studies strongly suggest that DNA pol η can be involved in the TLS of the abasic sites. Using purified human pol η, an efficient bypass of AP sites was reported and a preferential insertion of purines was found (13). The interaction between PCNA and pol η strongly increased its bypass ability and a more efficient extension reaction was observed from an A opposite the AP site than from a G opposite this lesion (14,19). More recently, Kokoska et al. (20) showed that human pol η and Sulfolobus solfataricus DNA polymerase 4 (Dpo4), a human pol κ homologue, efficiently bypassed abasic sites showing a strong bias for the adenine insertion.

To study the mutagenic potential of BER intermediates in mammalian cells we constructed single lesion-containing SV40-derived vectors presenting either an AP site or a 5′dRp or a 3′ddR5p sugar moiety at a defined position of their genome. The molecular nature of mutations was established after vector progeny rescue from simian cells. We were able to confirm the ‘A’ signature for abasic sites-induced mutations in mammalian cells and provide original evidence that AP sites derivatives 3′ or 5′ blocked ssb are also mutagenic. We hypothesise that restart of stalled forks can occur at BER intermediates thus leading to mutational events.

**MATERIALS AND METHODS**

**Chemicals**

Chemicals were purchased from Sigma. T4 DNA polymerase, T4 DNA ligase and T4 gene 32 protein were purchased from Roche Molecular Biochemicals. Restriction enzymes were from New England Biolabs.

**Preparation of DNA substrates**

The shuttle vector used in this study, pC2, is a derivative of phagemid pGEM-3Zf(+) and pSV-ori. To obtain pC2, the pGEM phagemid (Promega, Madison, WI) was digested with NdeI and Scal to release the f1 phage origin, which allows the rescue of single-stranded molecules. Before ligation step, the NdeI site was filled in with Klenow fragment enzyme. pSVori was digested with Scal and Accl; this fragment contains the SV40 replication origin necessary to allow the vector replication after transfection in COS cells.

Oligonucleotides for in vitro replication assay were purchased from Life Technologies. Closed circular DNA containing a single lesion was produced as described previously (21) by priming single-stranded (+) pGEM-3ZF DNA with 30-fold molar excess of lesion-containing oligonucleotide and incubating with T4 DNA polymerase holoenzyme, single-stranded DNA binding protein and T4 DNA ligase. Single-stranded DNA of the pGEM plasmid was produced according to the manufacturer’s instruction using the M13K07 helper phage. Closed circular DNA duplex molecules were purified by caesium chloride equilibrium centrifugation. The oligonucleotide 5′-GAT CCT CTA GAG TCG HX CC TGC A-3′ was used to create molecules containing a single hypoxanthine.
(HX) residue. The complementary sequence of single-stranded pGEM molecule was 5'-TGCAGGTCTGACTCTAGAGGATC-3'. By this construction scheme, the lesion is located on the lagging strand. Circular closed DNA molecules were digested with human monofunctional 3-methyladenine DNA glycosylase, the ANPG protein (a gift from Dr M. Saparbaev, CNRS, Institut Gustave Roussy, Villejuif Cedex, France) to produce a single AP site. 5'dRp and 3'ddRp5p residue-containing plasmids were created by APE 1 digestion (a gift from Dr I. D. Hickson, John Rodcliffe Hospital, University of Oxford, UK) and E.coli nth endonuclease (a gift from Dr S. Boiteux, Centre Energie Atomique, Fontenay aux Roses, France), respectively.

Cell culture and transfection

Monkey COS7 cells were grown as monolayers in DMEM (Gibco), supplemented with 10% fetal calf serum (Flow Laboratories), penicillin and streptomycin in 5% CO2 incubator (Gibco), supplemented with 10% fetal calf serum (Flow Laboratories), penicillin and streptomycin in 5% CO2 incubator (Gibco). The plasmid pC2 was introduced into the cells by the calcium phosphate coprecipitation technique and the precipitate was left on the cells overnight. Three days after transfection, the cells were collected and the SV40-derived shuttle vectors were recovered by Hirt procedure. To make sure that only plasmid molecules that replicated in mammalian cells were used as substrates for RFLP/PCR, the pC2 molecules recovered after transfection in COS cells were extensively digested by DpnI prior to amplification.

Selection for mutations in SalI recognition sequence of pC2 molecules by RFLP/PCR

In our shuttle vectors, the single lesion is located within the recognition sequence of specific restriction endonucleases (SalI/AccI/HincII) thus allowing recovery of mutants by restriction fragment length polymorphism PCR (RFLP/PCR) technique. An aliquot of 1 ng of pC2 DNA or aliquots of the shuttle vector DNA isolated from COS cells were extensively digested with the selected restriction endonuclease. Three rounds of 1 h digestion with 20 U of SalI were performed and the restricted DNA was purified by drop dialysis for 30 min at 4°C. The vector molecules resistant to cleavage were then amplified by PCR. The sequences of the primers were forward 5'—GCCAGTGAATTGTAA—GC-3' and reverse 5'—CTGTTGGATAACCGTATTACC-3'. The resulting PCR product contained the multiple cloning sites region of pC2, where the lesion of interest was initially located. PCR was performed by adding 2.5 U of Taq gold polymerase (Applied Biosystems) according to the standard conditions. The cycle profile was as follows: (i) 94°C for 1 min; (ii) 55°C for 2 min; (iii) 70°C for 3 min. After 20 cycles of amplification, a small aliquot (1/10) of the PCR was checked on agarose gel and, if necessary, other 10 cycles of amplification were applied. The amplified molecules were further digested and, if a partial contamination of sensitive/wild-type molecules was still present, the resistant/mutated molecules were separated on agarose gel and purified by gel purification kit (Qiagen). PCR molecules were cloned by TA cloning kit PCR II (Invitrogen) and sequenced. Mutants were sequenced with AmpliTaqFS on an ABI Prism 310 automatic sequencer (Perkin Elmer, Milano, Italy)

Quantitative PCR

Relative standard curve approach was employed and serial dilutions of pC2 DNA (dilution values from 10⁻³ to 10⁻⁹ ng) were used to construct a titration curve. For all experimental samples, ~1 ng of pC2 DNA, rescued by Hirt procedure after transient transfection of different adducted substrates, was amplified contextually to SalI-digested pC2 samples. All amplification reaction were carried out by the ABI PRISM 7700 Sequence Detection System. The sequences of the primers and probe were forward 5'-AATACGACTCACTATTGGCCAGCAAATTC-3', reverse 5'-TGACCATTAGCC-CCAAGCT-3' and probe 5'-CTCTAGAGGATC CCC-3'. The cycle profile was according to the manufacturer's instructions. According to the standard curve, the mean quantity of the non-digested and SalI-digested molecules was calculated automatically from software. To correctly estimate the mutation frequency of AP sites and derivatives, the Taq polymerase-induced mutation frequency was subtracted as background and the mutation frequency was calculated as follows: (number of mutated molecules/total number of molecules) × 100 (for details see Supplementary Figures 1 and 2).

RESULTS

Development of the experimental system

The experimental strategy is depicted in Figure 1A. pC2/HX plasmid molecules, which contain a single hypoxanthine (HX) within the SalI recognition sequence, were constructed by in vitro DNA replication. The characterization of the single lesion-containing vector was performed by constructing ³²P-labelled plasmid molecules, as described previously (22). The incubation of pC2/HX DNA with ANPG followed by digestion with E.coli endonuclease III revealed a complete conversion of covalently closed circular molecules to nicked form (data not shown), thus confirming that all molecules contained a single HX. The abasic site and its derivatives, 5' and 3' ssb (Figure 1B), were obtained by digestion of these molecules with the appropriate enzymes. The preparation of lesion-containing plasmids closely preceded cell transfection in order to minimize the problem of instability of BER intermediates. The single lesion-containing vectors were transiently transfected into simian cells and the vector progeny was isolated. The rescued pC2 DNA molecules were extensively digested with SalI and the molecules resistant to cleavage (mutant molecules) were selectively amplified by PCR. The amplified product was extracted from the gel, digested again with SalI and then subcloned into pCR II vector for sequence analysis. To calculate the mutation frequency induced by the different lesions a quantitative PCR (QPCR) analysis was performed.

In order to validate the system, two types of plasmid, one containing the unmodified SalI sequence (negative control) and one containing a single HX residue (positive control), were subjected to all the procedures, and mutation type and frequency were determined. Mutation analysis of the unmodified plasmid revealed the occurrence of point mutations with GC>AT transitions as predominant class (57%). Mutations were spread along the entire SalI recognition sequence.
The site where the lesion was originally built (the adenine of the SalI site) was a cold spot of mutation (Figure 2A). Mutation frequency was in the order of 0.4% (0.14 and 0.63% in two independent experiments) (Table 1). The type of mutations detected is in agreement with the mutational properties of Taq polymerase.

When the progeny of the HX-containing vectors was analysed, only point mutations, exclusively AT > GC transitions, were detected. The totality of mutation events mapped where the lesion was originally located (Figure 2B) and the mutation frequency was 7.2% (7 and 7.4% in two independent experiments). AT > GC transversions are indeed expected to arise following replication into mammalian cells of HX-containing vectors. The high mutagenic potential of this deaminated purine is in agreement with previous studies showing both a poor HX-DNA glycosylase activity in vivo and the high miscoding potential of this lesion (23).

**Mutation spectrum of BER intermediates**

*Abasic sites.* The specificity and sensitivity of our experimental strategy prompted us to investigate the miscoding properties of the AP sites in mammalian cells. pC2/HX molecules were incubated with ANPG immediately before transfection to generate shuttle vectors with a single natural AP site at a known position of their genome. Point mutations were the predominant event occurring at the AP sites (Figure 3A) in the rescued shuttle vector progeny. A small proportion (16%) of deletions was also observed, the majority spanning between 8 and 168 bp. Similarly to what observed in the case of HX, all point mutations mapped where the AP site was originally located. Among the point mutations, transversions were the predominant events (61%): both AT > TA (48%) and AT > CG (13%) base substitutions were recovered. The remaining mutations were AT > GC transitions. The mutation...
spectrum (Figure 3B) revealed that dAMP (48%) and dCMP (39%) were preferentially misincorporated opposite the AP site, whereas the incorporation of dGMP was a rare event. In our vector, the incorporation of TMP could not be detected since it restores the wild-type sequence. No frameshift events were found; however, it should be noticed that in our vector the abasic site is located in sequence context unfavourable to frameshift mutations.

The AP site mutation profile observed supports the so-called ‘A-rule’ model, i.e. the preferential insertion of an adenine opposite an AP site. The template realignment mechanism could also be evoked (see Discussion).

5’ dRp. During BER, the base loss sites are converted into ssb by the action of 5’AP endonucleases, which lead to the formation of 5’dRp and 3’ hydroxyl termini. In order to investigate the specific mutagenic properties of the 5’dRp intermediate, pC2/HX plasmid molecules, previously digested by ANPG and subsequently by APE 1, were transfected in COS cells. The molecular analysis of the 5’dRp-induced mutations revealed that the majority of events were point mutations (91%) with a high prevalence of transversions (87%) (Figure 4A). A small proportion (9%) of deletions, which were spanning between 1 and 39 bp, was also found. In contrast to what observed with the AP site, both dATP and dGTP were inserted opposite this intermediate with a similar efficiency (46 and 41% of the total events, respectively). A very low frequency of dCTP incorporation was observed (Figure 4B).

3’ ddR5p. Because of the instability of the aldehyde, AP sites can be spontaneously converted to ssb by β-elimination
reaction and give rise to 3′dR5p and 5′ phosphate termini. The same termini are generated by the action of the 3′lyase activity associated with bifunctional DNA glycosylases, such as OGG1, TDG, etc. In order to study the mutagenic potential of this 3′ modified terminus, pC2/HX plasmid molecules, previously digested by ANPG and subsequently by E.coli endonuclease III, were transfected in COS cells. Similarly to what observed with the 5′ssb, the mutation spectrum induced by this intermediate was characterized by the dominance of point mutations (61%), mainly transversions (78%) (Figure 5A). The analysis of the base inserted opposite the 3′dR5p showed a strong bias in favour of adenine, following the order dATP>dCTP = dGTP (55, 22.5 and 22.5%, respectively) (Figure 5B).

**Mutation frequencies at BER intermediates**

The mutagenic potential of the BER intermediates was evaluated by QPCR analysis. Table 1 summarizes the results of two independent experiments per each lesion. The mutation frequency detected at the SalI site of the undamaged and the HX-containing plasmids are shown for comparison.

Similar mutation frequencies were reported for AP, 5′dRp and 3′dR5P residues: 3.3, 3.7 and 4%, respectively.

The mutation frequency for the abasic site is in agreement with previously reported data (17). The high mutagenic potential of 5′ and 3′ ssb suggests that the persistence of these intermediates might be a threat for genome stability.

**DISCUSSION**

BER leads to the formation of several DNA repair intermediates that, if unsolved at replication, may threaten genomic stability by serving as blocks to replication, by stalling RNA polymerase and by promoting topoisomerase II-mediated double-strand breaks. In this study we have specifically addressed the mutagenicity of abasic sites and derivatives by using a single lesion-containing vector transfected into mammalian cells. We show that QPCR is a valuable tool to precisely quantify mutant rescue.

The molecular analysis of the mutations induced by natural AP sites showed that point mutations are the predominant events. A slight bias for the preferential insertion of an adenine was observed. These data are consistent with mechanistic studies of primer extension catalysed by purified mammalian DNA polymerases (16) and mutagenesis studies in both E.coli and mammalian cells (24). In particular, in mammalian cells, a preferential incorporation of an adenine opposite a synthetic abasic site was reported by replication of a single lesion-containing oligonucleotide (16) and, more recently, by a quantitative assay for TLS across a THF residue (25). One study (17) did not find any specific base incorporation opposite AP site. Sequence-specific effects might explain this discrepancy. Pioneer NMR spectroscopy studies on DNA duplexes containing a single AP site provide a possible mechanistic explanation for the ‘A-rule’. Structure analysis of these duplexes showed that only when an adenine was inserted, the base was within the double helix and DNA β-configuration was retained, while the insertion of a pyrimidine produced a DNA collapsed structure with the lesion situated out of the helix (26).

We observed that, after adenine, the most frequently misincorporated base opposite the AP site is a cytosine. A well-established model for AP site-induced mutagenesis predicts that replicative DNA polymerase might loop out the lesion and use the 5′ flanking base to the abasic site to instruct nucleotide incorporation, (′5′-rule′). According to this model, the nature of base substitution should be driven by the specific 5′ flanking base used as template. The high frequency of AT-GC transitions due to cytosine incorporation opposite the AP site observed in this study might be ascribed to this mechanism.

Which is the DNA polymerase involved in AP site bypass? Some lines of evidence suggest that human DNA pol η, first characterized for the error free bypass of cyclobutane pyrimidine dimers, might be involved. In particular, the order and the ratio of dNTPs insertion opposite an abasic site was G > A > C > T (51%-39%-6%-4%) while the frequency extension from different 3′-terminal dNTPs/AP site was A > C > G > T (52%-20%-18%-10%) (14). More recently, besides pol η, an archaeal Y-polymerase homologue of human pol κ, Dpo4, was shown to efficiently bypass the abasic sites by a strong preferential adenine incorporation (20,27).

All together, these data lead to a model where the replicative DNA polymerase stalled to the AP site could rarely manage to incorporate a base and, if it does, an adenine should be incorporated opposite the lesion (′A-rule′) but without any extension. This reaction should be accomplished by a TLS DNA polymerase, likely pol η or pol κ that synthesizes several nucleotides until resuming of regular replication (28). The AP site-induced mutation spectrum that others and we have reported in mammalian cells perfectly fits to this model.

Several reports suggested that natural abasic sites might also promote 1- and 2-base deletions (16,20). NMR studies...
clarified that a slipped intermediate with the lesion looped out of the helix could also arise after adenine incorporation but specific sequence context was required (a thymidine positioned 5' to abasic site) to stabilize such a structure and potentially to lead to frameshift mutations (29). The specific sequence constraint for AP site-induced frameshifts could justify the absence of this type of mutations in the present study.

The surprising finding of this study is the detection of point mutations after in vivo replication of vector molecules containing a single 5'dRp or 3'ddR5p residue. Mutation frequency was very high and comparable with that of AP sites (~3–4%). When the replication machinery encounters these termini, it is expected that a block of replication occurs leading to lethality (30,31). Our experimental system has not been designed to measure lethal events. Therefore, we cannot exclude that, if these termini act as strong blocks to replication, we could have an overestimation of the observed mutation frequency and a potential bias on mutation spectrum. Previous studies have addressed the mutagenic potential of ssb in human cells by using nicked pZ189 shuttle vector molecules as a tool (32). Base substitutions were predominantly detected and were expected that a block of replication occurs leading to lethality (30,31). Our experimental system has not been designed to measure lethal events. Therefore, we cannot exclude that, if these termini act as strong blocks to replication, we could have an overestimation of the observed mutation frequency and a potential bias on mutation spectrum. Previous studies have addressed the mutagenic potential of ssb in human cells by using nicked pZ189 shuttle vector molecules as a tool (32). Base substitutions were predominantly detected and were

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Conflict of interest statement. None declared.

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SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.
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