The Base Substitution Fidelity of DNA Polymerase β-dependent Single Nucleotide Base Excision Repair*

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Damaged DNA bases are removed from mammalian genomes by base excision repair (BER). Single nucleotide BER requires several enzymatic activities, including DNA polymerase and 5′,3′-deoxyribose-5-phosphate lyase. Both activities are intrinsic to four human DNA polymerases whose base substitution error rate during gap-filling DNA synthesis varies by more than 10,000-fold. This suggests that BER fidelity could vary over a wide range in an enzyme dependent manner. To investigate this possibility, here we describe an assay to measure the fidelity of BER reactions reconstituted with purified enzymes. When human uracil DNA glycosylase, AP endonuclease, DNA polymerase β, and DNA ligase 1 replace uracil opposite template A or G, base substitution error rates are ≤0.3 to ≤2.8 × 10⁻⁴. BER error rates are higher when excess incorrect dNTPs are included in the reaction or when wild type DNA polymerase β is replaced by DNA polymerase β variants that fill single nucleotide gaps with lower fidelity. Under these conditions, the base substitution fidelity of polymerase β-dependent BER is 3–8-fold higher than is single nucleotide gap filling by polymerase β alone. Thus other proteins in the BER reaction may enhance the base substitution fidelity of DNA polymerase β during single nucleotide BER.

Base excision repair (BER) protects cells against the lethal and mutagenic effects of DNA damage by correctly replacing damaged bases with undamaged bases. Mammalian cells harbor more than one BER pathway (1). One way to distinguish these is by the number of nucleotides replaced, which can be from one to more than 10. Single nucleotide BER is initiated upon recognition of an altered base by a monofunctional DNA glycosylase that cleaves the glycosylic bond to release the base and produce an apurinic/apyrimidinic (AP) site. The DNA backbone is cleaved 5′ to the AP site by a class II AP endonuclease (APE), producing a 5′,3′-deoxyribose-5-phosphate (dRP) and a 3′-hydroxyl terminus. The missing base is replaced by polymerase, the displaced dRP group is excised, and the resulting nick is ligated to complete repair.

Multiple proteins exist in mammalian cells that theoretically could participate in each step in BER. By virtue of its DNA polymerase and dRP lyase activities (2), DNA polymerase β (pol β) has a major role in single nucleotide BER of uracil in mammalian cells (3, 4). Three other human DNA polymerases also have dRP lyase activity, pol γ (5), pol ε (6), and pol λ (7), and pol λ can substitute for pol β in BER reactions in vitro that repair uracil paired opposite adenine or guanine (6, 7). Such enzymatic redundancy is interesting because the base substitution fidelity of these four DNA polymerases differs over a remarkable range when they work alone to fill gaps in DNA. For misincorporation of dGMP opposite template thymine, proofreading-proficient human mitochondrial pol γ has an error rate of 10⁻⁵ (8), human pol ε has an error rate of >10⁻⁶ (6, 9–11), and human pol β (12–14) and pol λ (15) have error rates between these two values. Moreover, while the latter three polymerases lack intrinsic exonuclease activity, human APE is reported to have 3′ to 5′ exonuclease activity that can excise nucleotides misinserted by pol β (16). Thus APE or an exonuclease such as TREX 1 (17, 18) may proofread errors made by exonuclease-deficient DNA polymerases during BER.

The number of DNA bases subject to BER due to modification by normal cellular processes like depurination, deamination, oxidation, and alkylation has been estimated to exceed 10,000 per day (19). Exposure of cells to physical and chemical agents in the environment can further increase the number of modified bases. Thus errors arising during BER could give rise to one or more mismatches per day that, if uncorrected, could result in base substitutions. For example, uracil-initiated BER in mammalian cell-free extracts involving replacing one to eight nucleotides generated substitution error frequencies ranging from 5.2 to 7.2 × 10⁻⁴ (20–22). That more than one DNA polymerase can generate BER errors in cell-free extracts was demonstrated by differences in BER error specificity in extracts of pol β (+/+ and −/−) mouse cells (22).

To investigate the enzymes and mechanisms responsible for determining the fidelity of BER pathways, here we describe an assay to determine error rates during BER reactions reconstituted with purified proteins. We measure base substitution error rates for repair of uracil opposite adenine or guanine, in single nucleotide BER reactions reconstituted with purified proteins, one of which is human pol β. The results establish the minimum fidelity of this single nucleotide BER reaction and indicate that BER fidelity is at least severalfold higher than the fidelity of single nucleotide gap filling DNA synthesis by pol β alone.

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¶ The abbreviations used are: BER, base excision repair; AP, apurinic/apyrimidinic; APE, AP endonuclease; dRP, 5′,3′-deoxyribose-5-phosphate; pol, polymerase; UDG, uracil DNA glycosylase.

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EXPERIMENTAL PROCEDURES

Materials—Materials for the fidelity assay were from previously described sources (23). Restriction endonucleases EcoRI, PstI, and T4 DNA ligase were from New England Biolabs. [γ-32P]dCTP, [γ-32P]dTTP, and dNTP were from Amersham Biosciences. High performance liquid chromatography-purified or gel-purified oligonucleotides were obtained from Oligos Etc., Inc. (Wilsonville, OR). Recombinant human uracil DNA glycosylase (UDG) with 84 amino acids deleted from the amino terminus, APE, pol, and DNA ligase I were purified as described previously (24).

Preparing Gapped DNA—Duplex DNA was formed by annealing two oligonucleotides (5'-AATTCCAGCTCGGTACCGGGCTAGCCTTTGGAGTCGACCTGCAGAAATT-3') and (3'-GGTCGAGCCATGGCCCGAGGTGGAAACCTCAGCTGGACGTCTTTAA-5'). The gapped DNA was prepared by hybridizing circular plus strand phage DNA to the minus strand of the large duplex fragment produced by an EcoRI/PstI double digestion of the double-stranded DNA, as described previously (23). The gapped DNA was separated by agarose gel electrophoresis and purified from gel slices by electroelution using an Elutrap (Schleicher and Schuell).

Substrates for BER—For experiments with a G:U substrate, a duplex was formed by annealing a 45-mer (5'-CCAGCTCGGTACCGGGCTAGCCTTTGGAGTCGACCTGCAGAAATT-3') and a 45-mer (3'-GGTCGAGCCATGGCCCGAGGTGGAAACCTCAGCTGGACGTCTTTAA-5'). For experiments with the A:U substrate, a duplex was prepared by annealing a 45-mer (5'-CCAGCTCGGTACCGGGCTAGCCTTTGGAGTCGACCTGCAGAAATT-3') and a 45-mer (3'-GGTCGAGCCATGGCCCGAGGTGGAAACCTCAGCTGGACGTCTTTAA-5').

In Vitro Base Excision Repair Assays—BER reactions were performed according to a previously described procedure (24) with modifications. The reaction mixture contained 4 pmol of uracil containing substrate, 50 mM Hepes, pH 7.5, 2 mM dithiothreitol, 0.2 mM EDTA, 100 μg/ml bovine serum albumin, and 10% glycerol. Reactions (12 μl) were initiated by adding 400 fmol of UDG and incubated at 37 °C for 5 min. The reaction (40 μl) was then supplemented with 5 mM MgCl2, 4 mM ATP, 10 mM APE, 10 mM pol β, 100 mM DNA ligase I or T4 DNA ligase (20 units/μl), 100 μM each of dATP, dCTP, dGTP, dTTP, and either 0.3 μM [γ-32P]dCTP (for G-U substrate) or 0.3 μM [γ-32P]dTTP (for A-U sub...
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Preparation of Repaired DNA—The repaired duplex DNA was purified by phenol/chloroform extraction followed by ethanol precipitation. Precipitated DNA was dissolved and the DNA was digested with PstI, followed by heat inactivation of PstI for 20 min at 80 °C. After adding an equal volume of gel loading buffer and incubating for 2 min at 95 °C, the DNA were separated by electrophoresis in a 12% polyacrylamide gel containing 7 M urea in 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.5. The repaired 35-mer oligonucleotides was located by autoradiography and excised, the gel slice was crushed and soaked in water, and the single-stranded oligonucleotide was recovered by ethanol precipitation. The amount of repaired DNA recovered was calculated from the radioactivity recovered, with estimated yields of about 50%.

Hybridizing the Repaired Oligonucleotide to Gapped DNA—The repaired (or unrepaird control) oligonucleotide was incubated for 3 min at 75 °C and then mixed with 10-fold molar excess with the gapped DNA in a buffer containing 300 mM NaCl, 30 mM sodium citrate. This mixture was incubated at 48 °C for 3 min, 42 °C for 10 min and then cooled to room temperature.

Measurement of Reversion Frequency—DNA samples were introduced into Escherichia coli MC1061 by electroporation, cells were plated, M13 plaque colors were scored, and reversion frequencies were calculated as described previously (23). DNA was isolated from collections of independent revertants to define the sequence change responsible for the dark blue plaque phenotype.

RESULTS AND DISCUSSION

Strategy to Measure BER Fidelity—The assay (Fig. 1A) monitors the fidelity of BER reactions that repair damage (e.g. uracil) present in a 45-base pair duplex DNA oligonucleotide. The DNA sequence (Fig. 1B) is a modified NH₂-terminal coding sequence of the lacZ α-complementation gene of bacteriophage M13mp2. Here we study incorporation of the correct base following removal of uracil paired with adenine (Fig. 1) or mis-paired with guanine. The adenine and guanine are located within a TAG codon. BER is performed with radiolabeled dNTP precursors, and a small amount of the reaction product is analyzed by electrophoresis to detect the 45-base pair product expected for complete single nucleotide BER. The remaining product is digested with PstI to generate a repaired, radiolabeled, 35-base pair minus strand and an unlabelled, 39-base pair, plus strand template. The minus and plus strands are separated by denaturing PAGE, and the repaired 35-base pair minus strand DNA is isolated free of its complementary strand. The repaired minus strand DNA is hybridized to circular, plus strand M13 DNA containing a 35-nucleotide single-stranded gap. This generates circular M13 molecules that express the repaired DNA strand when introduced into E. coli cells and plated to score M13 plaque colors. Correct incorporation of dTMP opposite A or dCMP opposite G during BER maintains the TAG codon. Although a counter stop codon in the NH₂ terminus of lacZ usually yields colorless M13 plaques (23), this particular TAG codon encodes a light blue plaques (24), presumably due to partial read through of the stop codon. Eight different base substitutions resulting from BER errors are detected as dark blue plaques (Fig. 1B). Incorporation of dTTP opposite G results in a TAA codon whose light blue phenotype is indistinguishable from that of the original TAG codon. Uracil that escapes BER will not increase the reversion frequency because it will yield a light blue plaque when paired with either the A or the G of the TAG codon. When 35-base pair oligonucleotides with substitutions at the adenine or guanine were hybridized to gapped circles containing the TAG codon and then introduced into E. coli cells, ~60% of the plaques arising from these mismatched heteroduplexes were dark blue. This is similar to previous studies showing an average of 60% expression of the minus strand in mismatched heteroduplexes (Ref. 23 and references therein) and indicates that errors made in vitro are expressed with high efficiency when introduced into E. coli cells.

Measurements of Single Nucleotide BER Fidelity—A BER reaction was performed with human UDG, APE, DNA pol β, and DNA ligase I. Reaction conditions were similar to those previously demonstrated to replace uracil paired opposite A or G with a single correct nucleotide (24). Control experiments (not shown) demonstrated that UDG removed >95% of the uracil present in the duplex substrate in the reaction. The DNA products of complete BER reactions included the expected 45 base pair ligated DNA characteristic of complete BER (Fig. 1C, lanes 2 and 3). Shorter chain length products were also observed that result from uracil removal, APE incision of the DNA backbone, and incorporation of the radiolabeled dNTP but without ligation. The 27-base pair product (Fig. 1C, lanes 1, 2, and 4) results from incorporation of one dNTP, while the slightly longer products result from limited strand displacement. Following digestion of the duplex products of BER with PstI, repaired 35-mer minus strand products (e.g. Fig. 1C, lane 5) were isolated and hybridized in 10-fold molar excess to gapped M13 DNA, and the reversion frequency of these circular M13 genomes was determined. Repair of uracil opposite template A in reactions containing correct dTTP but no incorrect nucleotides resulted in products whose reversion frequency was 3.4 × 10⁻⁴ (Table I, Experiment 1). The products of a BER reaction containing an equimolar concentration of all four dNTPs, thus providing the opportunity to misincorporate nucleotides, yielded a similar reversion frequency of 5.6 × 10⁻⁴. Both values are similar to the reversion frequency of 3.9 × 10⁻⁴ observed when an unrepaird uracil containing 35-mer was hybridized to the gap (Table I, line 2). Thus, pre-existing errors introduced during chemical synthesis of oligonucleotides contribute significantly to reversion frequencies in this range. Similar results were obtained for BER reactions that replaced uracil with dCTP opposite template G (Table I, Experiment 2).

DNA from independent blue plaque revertants were then sequenced. Among 24 revertants from reactions containing A-U duplex and only dTTP (Table II, Experiment 1), 19 contained a
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Error rates were calculated for each type of mismatch by subtracting the background revertant frequency for reactions containing only the correct dNTP, then dividing this value by 0.6, to correct for expression of the synthesis error in the nascent minus strand. For revertant frequencies after background subtraction that are less than or equal to the background value, or when no revertants of a particular type were observed, error rates are expressed as “less than or equal to” values.

### Table II
Revertant sequences, revertant frequencies, and error rates for BER

| DNA substrate | dNTPs or pol | Revertants/revertant frequency (×10⁻⁴) | Error rate (×10⁻⁴) |
|---------------|--------------|----------------------------------------|-------------------|
|               |              | Total | TGG (A-dCTP) | TGG (A-dATP) | TGG (A-dGTP) | TAT (G-dATP) | TAC (G-dGTP) |
| A–U           | dTTP         | 24    | 19/2.7       | 0/0.1        | 0/0.1        | –            | –            |
| Equal         | 24           | 173/9  | ≤2           | 4/0.1        | 0/0.2        | 0.3          | 0.7          |
| Biased        | 24           | 2/10/12| 1/0.4/0.7    | 0/0.4/0.7    | –            | –            | –            |
| G–U           | Equal        | 24    | –            | –            | –            | –            | –            |
| Biased        | 14           | –     | –            | –            | –            | –            | –            |
| A–U           | R283K        | 18    | 14/5/4/4.5   | 3/1.2/2.0    | 0/0.4/0.7    | 0/0.5        | 0/0.7        |
| R283A         | 14           | 11/51/81| 3/14/23     | 0/4.7/7.8    | –            | –            | –            |
| A–U           | R283K        | 14    | 11/10/12     | 1/0.9/1.5    | 0/0.9/1.5    | –            | –            |
| R283A         | 10           | 8/69/110| 1/8.7/15    | 0/8.7/15     | –            | –            | –            |

BER error rate with R283K was higher (4.5 × 10⁻⁴) than that observed with wild type pol β (≤2 × 10⁻⁴), consistent with the lower fidelity of R283K for the A-dCTP mismatch during gap filling (14). A BER reaction containing equimolar dNTPs and the R283A variant repaired the A-U substrate with error rates for the A-dATP and A-dGTP mismatches that were substantially different from the values observed with wild type pol β for these same mismatches (Table II, compare Experiments 1 and 3). This is consistent with the observation that the R283K replacement did not strongly increase error rates for these mismatches during gap filling (14). However, for the A-dCTP mismatch, the BER error rate with R283K was higher (4.5 × 10⁻⁴) than that observed with wild type pol β (≤2 × 10⁻⁴), consistent with the lower fidelity of R283K for the A-dCTP mismatch during gap filling (14). A BER reaction containing equimolar dNTPs and the R283A variant repaired the A-U substrate with error rates for the A-dCTP and A-dATP mismatches that were both more than 10-fold higher than observed with wild type pol β (Table II).

**Comparison of Error Rates for BER and Gap Filling by pol β Alone**—We previously determined error rates for wild type pol β and the R283K/A variants during synthesis to fill a gap containing a single template adenine (13, 14). Those studies employed equal concentrations of the four dNTPs and T4 DNA ligase to seal the nick following gap filling. To compare those results to BER fidelity, BER fidelity measurements were also performed with the R283K and R283A variants in reactions containing T4 DNA ligase rather than DNA ligase 1. The results were similar (Tables I and II, Experiment 4), suggesting that the ligase was not a critical determinant of fidelity under these conditions. We also measured error rates for wild type pol β during single base (adenine) gap-filling synthesis (14) in the presence of a 50-fold excess of dCTP, dATP, and dGTP over dTTP. The data allow comparison of the fidelity of pol β gap filling to the fidelity of pol β-dependent BER, under conditions in which BER generates one or more of the three possible substitution errors at rates above the background noise of the assay. The results show that BER error rates (Fig. 2, gray bars) are severalfold lower than error rates for gap filling by pol β alone (black bars). The differences suggest that the nucleotide selectivity of pol β may be enhanced during BER by the other proteins in the reaction, or perhaps by the presence of the 5’ dRP group, which is not excised until after polymerization has occurred. This could result from increased efficiency of correct nucleotide incorporation to enhance fidelity (26).
pend on the concentration of the next correct nucleotide. This could be advantageous for BER reactions that need to occur throughout the cell cycle regardless of whether dNTP concentrations are as high as in the S phase or following DNA damage (27) or as low as in the G phase. Also, once the mismatched DNA is generated, the competition for this nicked, mismatched DNA substrate is between the ligase active site and the exonuclease active site in two proteins separated in the single nucleotide BER reaction pathway by pol β. Substrate partitioning between proteins normally separated by two other enzymatic steps (polymerase and dRP lyase) represents a novel extension of the general idea that the product of one enzymatic step in BER is directly handed off to the next enzyme in the pathway (28, 29). That idea was put forth to explain how coordinating the BER step could avoid generating cytotoxic BER intermediates, whereas the current refinement would avoid not the cytotoxic but rather the mutagenic consequences of aberrant BER. Moreover, proofreading by a separate exonuclease may allow regulation of BER fidelity. For example, proofreading may suppress mutagenesis resulting from environmental stress, but it may be counterproductive for putative uracil-initiated BER reactions that could promote somatic hypermutation of immunoglobulin genes (reviewed in Ref. 30).

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Fig. 2. Error rates for single nucleotide BER and for gap filling by pol β alone. See "Results" for description. The error rates for formation of specific mismatches during BER (gray bars) are from Table II, and the error rates for formation of these same mismatches during gap filling by the R283A and R283K pol β variants (black bars) are from Osheroff (14). Wild type pol β alone was also used to fill a gap containing a single template A in the presence of 500 μM dCTP, dATP, and dGTP and 10 μM dTTP, otherwise as described in Ref. 14. The products of the reaction yielded a revertant frequency of 27 × 10⁻⁴ (602 blue plaques among 224,000 total plaques). Repeated experiments yielded revertant frequencies of 34 × 10⁻⁴ and 18 × 10⁻⁴. DNA sequence analysis showed that 40 of 41 revertants contained a single base substitution consistent with incorporation of dCMP opposite the template A. From this information and after correcting for expression of the nascent strand, the rate for this error (black bar on the left) was calculated to be 44 × 10⁻⁴.

Another possibility for the higher apparent fidelity of BER is proofreading by the reported 3′ to 5′ exonuclease activity of APE (16). The 3–8-fold differences observed (Fig. 2) are consistent with removal of 67–87% of mismisinserts. If this is due to APE proofreading, this efficiency is modest compared with the 90 to >99% efficiency of editing by most DNA polymerases with intrinsic exonucleases. In the latter cases, the contribution of proofreading to fidelity depends on the rate of excision of the misinserted nucleotide relative to the rate of extension of the mismatched primer terminus. For enzymes like pol δ and pol ε, the polymerase-exonuclease competition for the DNA substrate is between two active sites present in the same protein. Excision is usually strongly preferred over mismatch extension, whose rate is normally low and highly sensitive to the concentration of the next correct dNTP to be incorporated. However, proofreading of errors made by exonuclease-deficient polymerases like pol β during single nucleotide BER could differ in at least two ways. Since only a single nucleotide is incorporated, there is no need for further extension, so proofreading efficiency during single nucleotide BER may not de-
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