Fidelity and Mutational Specificity of Uracil-initiated Base Excision DNA Repair Synthesis in Human Glioblastoma Cell Extracts

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Russell J. Sanderson‡ and Dale W. Mosbaugh§§

From the ‡Departments of Agricultural Chemistry and Biochemistry and Biophysics and the §Environmental Health Science Center, Oregon State University, Corvallis, Oregon 97331

The fidelity of DNA synthesis associated with uracil-initiated base excision repair was measured in human whole cell extracts. An M13mp2 lacZ α DNA-based reversion assay was developed to assess the error frequency of DNA repair synthesis at a site-specific uracil residue. All three possible base substitution errors were detected at the uracil target causing reversion of opal codon 14 in the Escherichia coli lacZ gene. Using human glioblastoma U251 whole cell extracts, approximately 50% of the heteroduplex uracil-containing DNA substrate was completely repaired, as determined by the insensitivity of form I DNA reaction products to cleavage by a combined treatment of E. coli uracil-DNA glycosylase and endonuclease IV. The majority of repair occurred by the uracil-initiated base excision repair pathway, since the addition of the bacteriophage PBS2 uracil-DNA glycosylase inhibitor protein to extracts significantly blocked this process. In addition, the formation of repaired form I DNA molecules occurred concurrently with limited DNA synthesis, which was largely restricted to the HindIII DNA fragment initially containing the uracil residue and specific to the uracil-containing DNA strand. Based on the reversion frequency of repaired M13mp2 DNA, the fidelity of DNA repair synthesis at the target was determined to be about one misincorporated nucleotide per 1900 repaired uracil residues. The major class of base substitutions propagated transversion mutations, which were distributed almost equally between T to G and T to A changes in the template. A similar mutation frequency was also observed using whole cell extracts from human colon adenocarcinoma LoVo cells, suggesting that mismatch repair did not interfere with the fidelity measurements.

Uracil-initiated base excision repair (BER) maintains the genetic integrity of the human genome by removing uracil residues that accumulate in cellular DNA following the spontaneous deamination of cytosine in DNA or incorporation of dUMP during DNA synthesis (1). Unrepaired uracil residues in DNA contribute to cytotoxic, mutagenic, and lethal events in the cell (1, 2). The basic steps of uracil-initiated BER are conserved between bacterial and human cells, which underscores the biological significance of this repair pathway (for reviews, see Refs. 1, 3, and 4). Repair is initiated by hydrolytic cleavage of the N-glycosyl bond linking the uracil base to the deoxyribose phosphate DNA backbone by uracil-DNA glycosylase, releasing free uracil and producing an apyrimidinic site (5, 6). Incision of the phosphodiester bond on the 5′-side of the AP site by AP endonuclease generates a terminal 3′-hydroxyl-containing nucleotide and a deoxyribose 5′-phosphate moiety (7, 8), which is subsequently removed by DNA deoxyribophosphodiesterase to create a terminal 5′-phosphate-containing nucleotide and a single nucleotide gap (9–11). DNA polymerase mediates repair synthesis, and DNA ligase covalently links the phosphodiester backbone to complete the BER process (12–14). Uracil-initiated BER has been previously examined in cell-free extracts of Escherichia coli (15), Saccharomyces cerevisiae (16), hamster (17), bovine (18), and human cells (14, 15, 17).

Five distinct forms of uracil-DNA glycosylase have been previously reported in human cells; however, their relative contribution to BER remains to be determined in vivo. A mitochondrial (UNG1) and nuclear (UNG2) enzyme derived from the same UNG gene (also designated UDG1) by alternative mRNA splicing have been extensively studied (19, 20). These two subcellular forms apparently have identical catalytic domains but different N-terminal sequences (19, 20). A cyclin-like uracil-DNA glycosylase (UDG2) was identified that shares significant amino acid similarity with the cyclin A protein family but not with the UNG gene products (21). Glyceraldehyde-3-phosphate dehydrogenase has been purported to exhibit uracil-DNA glycosylase activity but only after subunit dissociation from the natural tetrameric to monomeric form (22). Finally, thymine-DNA glycosylase, which was originally identified as a G/T mismatch repair enzyme, has recently been shown to specifically excise uracil from duplex DNA containing G·U mismpairs but not A·U base pairs (23, 24). The thymine-DNA glycosylase amino acid sequence does not appear to be related to the other four proteins (25). The heretofore mentioned uracil-DNA glycosylases can be classified into two categories based on their inhibition by the bacteriophage PBS1 or -2 uracil-DNA glycosylase inhibitor protein (Ugi). Ugi forms a stable 1:1 complex with uracil-DNA glycosylase that is essentially irreversible under physiological conditions (26, 27). The nuclear (28), mitochondrial (29), and cyclin-like uracil-DNA glycosylases (30) are each inhibited by Ugi. In contrast, human erythrocyte glyceraldehyde-3-phosphate dehydrogenase has not been found to bind Ugi, and neither HeLa nor human recombinant thymine-DNA glycosylase have been shown to be inhibited by Ugi (23, 24).

Recent studies suggest that following the removal of the uracil base and cleavage at the resultant AP site, two alternative pathways may be utilized for completing uracil-initiated BER in eukaryotic cells (17, 31). These pathways are distinguished by the size of the repair patch created during the DNA

‡ To whom correspondence should be addressed. Tel.: 541-737-1797; Fax: 541-737-0497.

§ The abbreviations used are: BER, base excision repair; AP, apurinic/apyrimidinic; bp, base pair(s); pol β, polymerase β; Endo IV, endonuclease IV.

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synthesis step. Short-patch BER results in a one-nucleotide incorporation event during DNA repair synthesis, whereas long patch BER involves repair synthesis of 2–7 nucleotides (15–18, 31, 32). Unlike short patch repair, long patch BER most likely depends on strand displacement and/or exonuclease activity to carry out additional DNA synthesis. While both pathways may operate in vivo, the primary pathway used by human cell extracts to remove uracil from DNA is reportedly the short patch BER mechanism (32, 33).

Several observations have implicated DNA polymerase β (pol β) as the predominant polymerase involved in uracil-initiated BER synthesis in eukaryotic cells. First, the biochemical properties of pol β are consistent with a gap-filling role during both short and long patch BER synthesis. The preferred DNA substrate of pol β is a 5'-phosphorylated one-nucleotide gap, which is formed as an intermediate in short-patch BER, although small gaps (2–6 nucleotides) may also be filled to completion (12, 34, 35). Second, the addition of purified pol β to human nuclear extracts increased uracil-initiated BER by as much as 10-fold, whereas DNA polymerase α, δ, and ε had no significant effect (18). Third, the observation that both human and Xenopus laevis DNA pol β possess an intrinsic deoxyribophosphodiesterase activity provides evidence that pol β directly participates in two consecutive steps of the BER pathway (9–11). Fourth, a recent study demonstrates that pol β is coordinately assembled onto AP site-containing DNA by human AP endonuclease bound to an uncleaved AP site (36). Fifth, evidence that pol β participates in a concordant BER pathway was suggested by the isolation of a bovine multiprotein complex containing UDG, AP endonuclease, pol β, and DNA ligase I, which was capable of conducting short patch BER in vitro (37). Sixth, reconstitution experiments using purified human proteins revealed that a complex between pol β, DNA ligase III, and XRCC1 may alternatively act to promote uracil-initiated short patch BER, since XRCC1 suppresses the limited DNA strand displacement activity of pol β (38). Recently, a long patch BER system was reconstituted using purified human AP endonuclease, Pol β, DNase IV, and DNA ligase I or III with a reduced AP site containing DNA substrate (32). In this case, the 5’ to 3’ exonuclease activity associated with DNAse IV was stimulated by proliferating cell nuclear antigen and facilitated larger gap formation (32). In vitro, long patch BER was reconstituted with either DNA polymerase β or δ; however, the addition of neutralizing polyclonal pol β-specific antibodies to human cell-free extracts caused a 20-fold reduction in the extent of long patch BER (32). Similar investigations using antibodies raised against either pol β or an amino-terminal fragment (8-kDa domain) of pol β appeared to completely inhibit uracil-initiated BER in bovine testis nuclear extracts (18). Collectively, these observations provide strong evidence that pol β plays a major role in uracil-initiated BER.

Although the molecular mechanism of human uracil-initiated BER is understood in some detail, the fidelity and error specificity of repair DNA synthesis during this process has not been adequately investigated. We have therefore developed an M13mp2 lacZa DNA-based reversion assay for detecting base substitution errors produced during BER in human whole cell extracts. The fidelity of BER synthesis at a defined uracil residue located in the E. coli lacZa gene of heteroduplex M13mp2 DNA was measured, and the mutation spectrum was elucidated.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides GGGTAACCGCGGTTTCCCCAGT-CACGTCACTAAAGAAC (A-41-mer) and GCACCTCAGCGCGTTT-CGCC (sequencing primer) were synthesized on an Applied Biosystems 380B DNA synthesizer by the Center for Gene Research and Biotechnology (Oregon State University). Two complementary oligonucleotides, AGCTTGCGTCAGTTUGGACGATTCCCGGGAAAT (U-34-mer) and AATTTCGGGAGTCTGCAGGCGACCAAGCT (A-34-mer), were synthesized by Midland Certified Reagent Company. Oligonucleotides CCGCTACGCTTCTTGTAAGAAC (U-23-mer) and CCGAAGCCTG-CTTGTTGTTTCTGCG (A-23-mer) were synthesized and gel purified by Oligos Etc. Deblocked, degenerated, and 5'-end-phosphorylated oligonucleotides were prepared as described previously (39, 40). E. coli strains CJ236, MC1061, NR9162, and CSH50 were provided by T. A. Kunkel (NIEHS, National Institutes of Health), and E. coli JM109 was obtained from New England Biolabs. E. coli RR1 containing pTL43W, an overexpression vector for producing T4 DNA polymerase, was provided by W. H. Konigsberg (Yale University). The established human glioblastoma U251 cell line was obtained from E. Radany (University of Michigan), and the human colon adenocarcinoma LoVo cell line was procured from the American Type Culture Collection. E. coli uracil-DNA glycosylase (fraction V) and Ugi (fraction IV) were purified as described by Sanderson and Mosbaugh (41). E. coli endonuclease IV (fraction V) was provided by D. Demple (Harvard University). T4 DNA polymerase was obtained from New England Biolabs or purified by the procedure of Rush and Konigsberg (42) except that a single-stranded DNA-agarose column was used in place of the DNA-cellulose column and the 32P-Affi-Gel 10 column was omitted. T4 polynucleotide kinase; restriction endonucleases EcoRI, SmalI, and HindIII; and T4 DNA ligase were purchased from New England Biolabs.

**Reaction Products**—The DNA was isolated from E. coli CJ236 cells and purified as described by Kunkel (43). Site-specific mutations in the lacZa gene were introduced by annealing 5'-end-phosphorylated oligonucleotide A-41-mer to M13mp2 DNA and conducting a primer extension reaction (43). Oligo-nucleotide A-41-mer shared sequence complementarity with lacZa from position 68 to 106 except for discrepancies at positions 78–80 and 98. These two sites introduced an opal codon (TGA) in place of an arginine codon (CGT) coding for amino acid residue 14 of the lacZa gene product and a silent mutation generating a unique Smal restriction site, respectively. Primer extension reaction mixtures (300 μl) contained 20 mM Hepes-KOH (pH 7.4); 2 mM dithiothreitol; 13 mM MgCl2, 1 mM ATP; a 500 μM concentration each of dATP, dTTP, dGTP, and dCTP; and 7.5 μg/ml acetylated bovine serum albumin; 27 units of T4 DNA polymerase; 1200 units of T4 DNA ligase; and 8.8 pmol of heteroduplex A-41-mer M13mp2 DNA. After incubation for 5 min on ice, 5 min at 25 °C, and 4 h at 37 °C, the reaction was terminated with the addition of 53 μl of 0.1 M EDTA. Competent E. coli JM109 cells (~1.4 × 1011 cells/ml) in 10% (w/v) glycerol (40 μl) were mixed with the primer-extended DNA (0.5 μl), and electroporation was carried out using a Gene Pulser system (Bio-Rad). After a single pulse at 2.5 kV, 25-microfarad capacitance, and 200 ohms. Transfected cells were diluted into SOC media (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KC1, 10 mM MgCl2, 20 μM glucose) and grown on M9 plates containing 0.4 μg/ml isopropyl-D-galactopyranosidase and 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranosidase. An isolated clear plaque was selected and plaque-purified. Plage were propagated in E. coli JM109 cells and M13mp2 DNA was purified (43). DNA sequence analysis was conducted using an Applied Biosystems 373 DNA sequencer by the Center for Gene Research and Biotechnology (Oregon State University) and verified that M13mp2op14 DNA contained the desired lacZa opal codon and a unique Smal restriction site.

**Preparation of Base Excision Repair DNA Substrates**—M13mp2op14 DNA was annealed to 5'-end phosphorylated oligonucleotides U-23-mer or A-23-mer, and primer extension reactions were performed similarly to that described above (40, 43). Covalently closed circular duplex DNA reaction products were isolated by ethidium bromide-cesium chloride gradient centrifugation as described by Sambrook et al. (44). Centrifugation was performed in a SW41 rotor (Beckman) at 39,000 rpm for 60 h at 20 °C. Form I DNA was isolated, extracted four times with an equal volume of 1-butanol saturated with 5 μl NaCl, concentrated using a Centricon-30 (Amicon) concentrator, and buffer-exchanged into TE buffer (10 μM Tris-HCl (pH 8.0), 1 mM EDTA).

**Preparation of Whole Cell Extracts**—Human glioblastoma U251 cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mg/ml NaHCO3, 0.3 mg/ml l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Human colon adenocarcinoma LoVo cells were grown in Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 20% fetal bovine serum and 1.2 mg/ml NaHCO3. All cell cultures were incubated at 37 °C, 5% CO2, and 90% humidity. Cells were grown to ~85% confluency in 75-cm2 tissue culture flasks and were harvested by scraping into 8 ml of phosphate-buffered saline (8.1 mM NaHPO4, 1.5 mM KH2PO4, 2.7 mM KC1, 137 mM NaCl (pH 7.2)). Whole cell extracts were prepared as...
described previously by Wood et al. (45).

**Base Excision DNA Repair Reactions—**Standard BER reactions mixtures contained 100 mU Tris-\( \text{HCl} \) (pH 7.5), 5 mM MgCl\(_2\), 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM β-NAD, 20 μM each of dATP, dGTP, and dCTP, 5 mM phosphocreatine di-\( \text{HCl} \), 20 units of phosphocreatine kinase, 10 mU T\(_{1}\) RNase, and 10 mU of form I homoduplex DNA (form I) or M13mp2op14 (AT) homoduplex DNA (form I), and 2 mg/ml whole cell extract protein. In some cases, 200 μCi/ml of [\( ^{32}\)P]dATP (6000 Ci/mmol) was also included in the reaction. Incubation occurred at 30 °C for various amounts of time. Reactions were terminated by the addition of 2000 units of Ugi and adjustment to 20 mM Tris/\( \text{HCl} \), pH 8.0. RNase A was then added to 80 μg/ml and incubated at 37 °C for 10 min. Following the addition of SDS to 0.5%, proteinase K was added to 190 μg/ml, and the mixture was incubated for an additional 30 min at 37 °C. The samples were extracted with an equal volume of phenol/chloroform/isooctyl alcohol (25:24:1), ethanol-precipitated, washed with 75% ethanol, and resuspended in 30 μl of TE buffer.

**Isolation of Repaired DNA—**Samples of repaired M13mp2op14 (UT) DNA, isolated as described above, were treated with 400 units of Ung per μg of DNA for 30 min at 37 °C. After terminating the reaction with a 10-fold unit excess of Ugi, 4 units of E. coli endonuclease IV (Endo IV) were added per μg of DNA, and incubation continued at 37 °C for 30 min. This reaction was then terminated by heating at 70 °C for 3 min. Form I DNA that was insensitive to the Ugi/Endo IV cleavage was isolated as described above. Briefly, DNA samples were combined with agarose gel buffer to a final concentration of 0.1% SDS, 10 mM EDTA, 5% (w/v) glycerol, and 0.01% bromphenol blue. After loading each sample (10 μl) onto an agarose gel (12 × 13 cm) containing TAE buffer (40 mM Tris acetate, 1 mM EDTA (pH 8.0)) and 0.1 μg/ml ethidium bromide, electrophoresis was performed at 100 V until the tracking dye migrated ~75% of the distance through the gel. Form I DNA was recovered from gel slices by electroelution into TAE buffer using an Elutrap (Schleicher and Schuell) apparatus, concentrated using a Centricon 30 (Amicon) concentrator, and buffer-exchanged with distilled water.

**Transfection of E. coli and Determination of Reversion Frequencies—**E. coli MC1061 or NR9162 cells were transfected with purified form I DNA as described previously by Roberts et al. (46). Briefly, form I DNA samples (0.5–2.5 μl) were added to competent E. coli cells (50 μl), and transfection was conducted using a Gene Pulser electroporation system with a single pulse at 2.0 kV, 25-microfarad capacitance, and 400 ohms. Transfected cells were diluted into SOC media, mixed with midlog E. coli CSH50 cells, plated on M9 plates containing 0.4 mM isopropyl \( β\)-thiogalactopyranoside and 1 mM/5-bromo-4-chloro-3-indolyl-\( β\)-D-galactopyranoside, and grown overnight at 37 °C. After scoring the plaques as either colorless or blue, the reversion frequency was calculated from the ratio of the number of blue plaques to total (colorless plus blue) plaques detected. Blue plaques were picked and placed into 200 μl of sterile 0.9% NaCl solution, and extracted phage were diluted into SM media and titrated against midlog E. coli CSH50 cells (50 μl). The results are shown in Table 1.

**Analysis of Base Excision Repair Reaction Products—**Standard BER reactions were performed except that 200 μCi/ml [\( ^{32}\)P]dATP (6000 Ci/mmol) was included in the reaction mixture. DNA reaction products were isolated and treated in the presence or absence of Ung/Endo IV, and form I and II DNA were resolved by 5% agarose gel electrophoresis as described above. Ethidium bromide-stained DNA bands were visualized by transillumination (302 nm), and the percentage of form I and II DNA was quantitated against form I and II DNA standards from the same gel using a gel documentation system (model GDS7500; Ultra-Violet Products Ltd.) and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The amount of form I and II DNA recovered after incubation with whole cell extract protein decreased in a time-dependent manner. The percentage of form I DNA measured was calculated from the total amount of form I and II detected in any given time point. To detect [\( ^{32}\)P]DNA, the gel was blotted onto a Gene Screen Plus (NEN Life Science Products) membrane using a downward alkaline transfer technique described by Koetsier et al. (47). Autoradiography was conducted using X-Omat(R) Eastman Kodak Co. film, and quantitation of form I and II [\( ^{32}\)P]DNA bands was performed using a PhosphorImager (Molecular Dynamics) and ImageQuant software.

[\( ^{32}\)P]DNA not treated with Ung/Endo IV was digested with HindIII restriction endonuclease, and restriction fragments were resolved by 5% nondenaturing polyacrylamide gel electrophoresis (39). After drying the gel, autoradiography was performed, and the amount of each [\( ^{32}\)P]DNA fragment was quantitated using a PhosphorImager. The relative intensity of various bands was determined, and the amount of [\( ^{32}\)P]DNA fragments (253, 261, 486, and 529 bp) was compared after subtracting background values obtained from a blank lane.

**Analysis of DNA Strand Specificity of DNA Synthesis—**Standard BER reactions were performed with M13mp2(\( T \)) homoduplex DNA, [\( ^{32}\)P]dATP as described above. DNA reaction products and simultaneously digested with EcoRI and Smal (10 units/μg of DNA) for 1 h at 25 °C. The restriction endonuclease reactions (10 μl) were terminated by adjustment to 12.2 mM EDTA and then treated with or without E. coli Ung and Endo IV as indicated. Samples were combined with an equal volume of denaturing formamide dye buffer (95% deionized formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol) and heated for 3 min at 95 °C, and DNA fragments were resolved by 12% polyacrylamide, 8.3 μm urea gel electrophoresis as described previously (39).

**Determination of Mutational Spectrum—**Isolated blue plaques obtained from the secondary screening were used to isolate M13mp2op14 derived single-stranded DNA as described previously (43). DNA sequence analysis was performed using the dideoxynucleotide chain termination method. The nucleotide sequence of the lac\( Z\)a gene was determined using a DNA primer complementary to the (−)-strand at nucleotide positions 249−269.

**RESULTS**

**Base Excision DNA Repair Assay—**An M13mp2 lac\( Z\)a DNA-based reversion assay was developed to investigate the fidelity of DNA synthesis associated with uracil-initiated DNA repair in human whole cell extracts as illustrated in Fig. 1. Site-directed mutagenesis was used to construct the base excision repair DNA substrate (M13mp2op14 DNA) containing a transcriptional stop codon in the E. coli lac\( Z\)a gene of M13mp2 DNA. By introducing an opal codon (TGA) in place of the arginine codon (CGT) for amino acid 14 of the lac\( Z\)a gene product, lac\( Z\)a was rendered inactive and incapable of α-complementation. Circular heteroduplex M13mp2op14 DNA with a site-specific uracil at nucleotide position 78 of the (−)-strand was then constructed, and form I DNA was purified. This DNA substrate contained a U78 derived strand mispair at the first nucleotide of the opal codon and served as the uracil target for repair. The uracil residue was strategically located so that faithful and unfaithful uracil-initiated DNA repair synthesis in human whole cell extracts could be distinguished based on reversion of the opal codon. If faithful DNA synthesis occurred with dAMP incorporation opposite thymine at nucleotide position 78, the opal codon sequence would be restored to the (−)-strand (Fig. 1C). Alternatively, if unfaithful DNA synthesis incorporated dGMP or dTMP, a wild type arginine codon would be generated, whereas dCMP incorporation would introduce a glycine codon into the (−)-strand. In order to preserve mispairs introduced into repaired M13mp2op14 DNA by inaccurate DNA synthesis, the substrate was transfected into E. coli NR9162 (mutS), which is methyl-directed mismatch repair-defective. Upon transfection of E. coli NR9162 (mutS) cells with faithfully repaired M13mp2op14 DNA, a colorless plaque was expected on host indicator plates due to the inability of the lac\( Z\)a (opal) gene product to perform α-complementation. In contrast, unfaithful incorporation of a noncomplementary nucleotide in place of the uracil residue restored α-complementation and produced mixed burst blue plaques (colorless plus blue). These phenotypes were independently verified by transfecting E. coli JM109 with M13mp2 DNA containing each of the four possible nucleotide substitutions at position 78 (data not shown). As expected, M13mp2 DNA containing a lac\( Z\)a opal (TGA) and arginine (CGA, AGA) codon 14 produced colorless

2 M13mp2 heteroduplex DNAs containing both an opal codon in the (−)-strand and a C, G, or T base at position 78 in the (−)-strand, are capable of expressing the (−)-strand phenotype with an efficiency of ~30% (48), thus resulting in mixed bursts.
and dark blue plaques, respectively; however, a glycine (GGA) codon was found to produce a light blue plaque. These results validated the assay for detecting misincorporation events introduced at the target site during uracil-initiated DNA repair synthesis.

Detection of Uracil-DNA Repair in Human Glioblastoma U251 Cell Extracts—Initial experiments were conducted using M13mp2op14 DNA to detect uracil-DNA repair in human U251 whole cell extracts. Form I DNA was mixed with whole cell extracts and incubated for various times (0, 15, and 60 min), DNA was isolated, and reaction products were analyzed by agarose gel electrophoresis (Fig. 2). Whereas the unreacted control DNA substrate was found to contain almost exclusively (≥95%) form I DNA, the amount of form I DNA was significantly reduced after incubation for 15 min with human whole cell extract. The majority of the DNA migrated as form II DNA molecules. While some form II DNA remained after further incubation (60 min), the amount of form I DNA dramatically increased, suggesting that DNA repair had occurred. To establish whether the uracil residue was removed during this process, the DNA reaction products were treated with excess E. coli uracil-DNA glycosylase and endonuclease IV in order to cleave uracil-containing form I DNA. Following this treatment, most of the form I DNA was shown to be resistant to Ung/Endo IV-mediated cleavage after the 60-min repair reaction. Thus, the uracil residue was apparently removed from a significant fraction of the M13mp2op14 DNA substrate.

Evidence for Uracil-initiated Base Excision DNA Repair in Human U251 Cell Extracts—To measure the overall rate of uracil-DNA repair and to assess the involvement of DNA repair synthesis, the M13mp2op14 DNA substrate was incubated for various times with human U251 whole cell extract in the presence of [α-32P]dATP. As before, the recovered DNA was treated with excess Ung and Endo IV prior to resolving the reaction products on a 0.8% agarose gel (Fig. 3A, lanes C— and D). Analysis of the ethidium bromide-stained gel revealed the time-dependent appearance of an Ung/Endo IV-resistant form I DNA band. The percentage of form I DNA detected in each lane was determined and is represented in Fig. 3B. As a control, the extent of Ung/Endo IV cleavage of the M13mp2op14 DNA substrate was examined (Fig. 3A, lanes C and D). Before Ung/Endo IV treatment, ~95% of the DNA migrated as form I, whereas after treatment, no detectable form I DNA was observed (Fig. 3B, columns C— and +). Thus, the substrate appeared to be completely susceptible to Ung/Endo IV cleavage. Upon examination of the products from the reaction time course, a linear rate (~4.8 fmol/min) for the appearance of Ung/Endo IV-resistant form I DNA was observed during the initial 30-min period. Thereafter, the rate appeared to plateau with ~50% of the DNA being re-
paired. Autoradiography of the agarose gel demonstrated that the Ung/Endo IV-resistant form I DNA appeared with concomitant incorporation of [$^{32}$P]dAMP (Fig. 3C). Interestingly, significantly less [$^{32}$P]dAMP incorporation was observed in the form I DNA as compared with form II DNA, although both represented 50% of the total DNA molecules after a 60-min BER reaction (Fig. 3C, lane 6). This result was expected if form I DNA was generated by BER involving DNA synthesis of only one or a few nucleotides. While these results imply that uracil-DNA repair involved limited DNA synthesis, these observations fail short of establishing that repair was uracil-initiated through the base excision repair pathway.

To determine if base excision repair was involved, Ugi protein was added to the whole cell extract in order to specifically inactivate human uracil-DNA glycosylase. In the absence of Ugi, U251 whole cell extracts contained 8.6 units of Ung/mg of extract protein. However, the addition of a 45-fold unit excess of Ugi over Ung reduced the level of Ung activity to the limit of detection in the in vitro assay. When the base excision repair assay was repeated in the presence of excess Ugi, the vast majority of recovered form I DNA was sensitive to Ung/Endo IV cleavage (Fig. 3D). This indicated that a Ugi-sensitive uracil-DNA glycosylase was initiating most of the repair and strongly suggests the involvement of the BER pathway. However, a small amount of form I DNA corresponding to 3.7 and 9.4% of the substrate was insensitive to Ung/Endo IV cleavage after 60 and 90 min of BER reaction, respectively. This implies that some repair may have occurred without the involvement of a Ugi-sensitive uracil-DNA glycosylase.

Analysis of Uracil-initiated DNA Repair Synthesis—In order to localize the repair synthesis on M13mp2op14 DNA, [$^{32}$P]dATP was added to human U251 whole cell extracts, and standard BER reactions were performed. At various times, reaction products were isolated and digested with the restriction endonuclease HinII, generating a 529-bp DNA fragment that contained the uracil target (Fig. 4A). Following the HinII digest, DNA fragments were resolved by nondenaturing polyacrylamide gel electrophoresis and autoradiography was performed to detect [$^{32}$P]dAMP incorporation (Fig. 4B). While [$^{32}$P]dAMP incorporation was detected in many of the fragments, the 529-bp fragment showed preferential incorporation that accumulated in a time-dependent manner. Control reactions were conducted with both M13mp2op14 DNA containing an AT base pair or U/T mispair at position 78 of the lacZ gene to determine whether incorporation into other fragments resulted from nonspecific DNA synthesis (Fig. 4C). As before, preferential incorporation was observed specifically into the 529-bp fragment but only when containing the uracil residue. Corresponding non-uracil-containing fragments between both

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*Fig. 2. Agarose gel electrophoresis of M13mp2op14 DNA following base excision DNA repair in human U251 cell extracts. A standard BER reaction mixture (600 μl) containing 6 μg of M13mp2op14 (U/T) DNA (form I) and 1.2 mg of human U251 whole cell extract protein was incubated at 30 °C, and aliquots (150 μl) were removed at various times (0, 15, and 60 min). DNA reaction products were then isolated, treated with (+) or without (−) E. coli Ung and Endo IV, and analyzed by 0.8% agarose gel electrophoresis as described under “Experimental Procedures.” The position of form I and II DNA is indicated by horizontal arrows. Form I DNA that was resistant to Ung/Endo IV treatment was isolated from the gel slice (bracket) by electroelution into 1 ml of TAE buffer and concentrated (∼5-fold) using a Centricon 30 concentrator.*

*Fig. 3. Analysis of reaction products generated by uracil-DNA base excision repair in human U251 cell extracts. A, a standard BER reaction mixture (1050 μl) containing 10.5 μg of M13mp2op14 (U/T) DNA, 2.1 mg of U251 whole cell extract protein, and 210 μCi of [$^{32}$P]dATP was prepared. Samples (150 μl) were removed after incubation for 0, 15, 30, 45, 60, and 90 min at 30 °C (lanes 2–7, respectively), and each reaction was terminated by the addition of 2000 units of Ugi followed by adjustment to 20 mM EDTA. DNA was isolated, treated with E. coli Ung and Endo IV, and prepared for 0.8% agarose gel electrophoresis as described under “Experimental Procedures.” As a control, M13mp2op14 (U/T) DNA (1.5 μg) was mock-treated without cell extract and incubated with Ung and Endo IV (lane 1). Untreated M13mp2op14 (U/T) DNA (25 ng) and a sample containing 2.5 μg of a 1-kilobase pair DNA ladder (Life Technologies, Inc.) were analyzed as reference standards (lanes C and M, respectively). The location of ethidium bromide-stained form I and II DNA bands are indicated by arrows. B, DNA bands detected by ethidium bromide staining (A) were quantitatively measured using a gel documentation system, and the percentage of form I DNA in each sample was determined. The amounts of form I and II DNA were measured relative to standards (6.3–100 ng) analyzed on the same gel. The percentage of form I DNA observed before (−) or after (+) E. coli Ung/Endo IV treatment was determined after correcting for the ∼0.38-fold reduced ethidium bromide staining intensity of form I compared with form II DNA. The percentage of form I DNA detected was calculated by dividing the amount of form I DNA (ng) by that of form I plus II DNA and multiplying by 100. C, autoradiography was conducted after blotting [$^{32}$P]DNA from the 0.8% agarose gel onto a Gene Screen Plus membrane. D, a standard reaction mixture (600 μl) was prepared as described under “Experimental Procedures” except that 1000 units of Ung was added before the addition of substrate. Samples (150 μl) were removed at various times, treated with (+) or without (−) E. coli Ung/Endo IV as indicated, and processed for gel electrophoresis, and data were analyzed as described above.*

*The limit of detection for the standard uracil-DNA glycosylase assay was estimated to be approximately 0.001 units.*
substrates appeared to accumulate similar levels of $[^{32}P]$dAMP incorporation. The level of specificity was evaluated after quantifying the amount of $[^{32}P]$dAMP incorporation into individual DNA fragments (Fig. 4D). During the reaction time course, the relative amount of incorporation was compared for four DNA fragments (253, 529, 261, and 486 bp). A 5.2–7.6-fold greater $[^{32}P]$dAMP incorporation was observed on the U-529 fragment than on a similar sized 486-bp fragment located on the opposite side of the M13map2op14 DNA molecule. Similarly, only low levels of incorporation were detected for the 253- and 261-bp fragments that flanked either side of the uracil-containing 529-bp DNA fragment. When taken together, these results indicate that the vast majority of DNA synthesis on the 529-bp fragment was uracil-initiated and was limited to this fragment. Thus, most of the DNA synthesis observed was consistent with the occurrence of a uracil-initiated base excision repair mechanism.

DNA repair synthesis was further characterized to determine whether $[^{32}P]$dAMP incorporation occurred on the (−)-strand, (+)-strand, or both DNA strands. Standard BER reactions were conducted in the presence of [α-$^{32}$P]dATP, and DNA reaction products were then digested with restriction endonucleases EcoRI and SmaI. As illustrated in Fig. 1A, the resulting restriction fragment contained a 44- and 40-nucleotide sequence corresponding to the DNA (+)- and (−)-strands, respectively. This allowed the uracil-containing (−)-strand to be distinguished from the (+)-strand based on the size of the single-stranded DNA fragments. When restriction endonuclease-digested DNA products were analyzed by denaturing polyacrylamide gel electrophoresis, incorporation of $[^{32}P]$dAMP was observed to accumulate during the time course in association with the 40-mer fragment (Fig. 5A, lanes 1–6, left). No incorporation was observed into the 44-mer fragment. To determine whether the uracil residue was removed from the 40-mer during DNA repair synthesis, a duplicate set of $[^{32}P]$DNA samples was treated with E. coli Ung and Endo IV following restriction endonuclease digestions. As before, $[^{32}P]$dAMP incorporation was observed in the 40-mer at nearly the same level, and no smaller $^{32}$P-labeled fragments were detected as a result of cleavage at the uracil site (Fig. 5A, lanes 1–6, right). As a control, the activity of both Ung and Endo IV was verified using a duplex uracil-containing 34-mer substrate (Fig. 5B). Collectively, these results indicate that DNA repair synthesis was directed exclusively to the (−)-strand containing the uracil target.
of conducting methyl-directed mismatch repair. Direct determination of the background reversion frequency for M13mp2op14 (U/T) DNA was not possible, since the (−)-strand DNA correctly encodes a wild type lacZa gene. Hence, unrepaired form I DNA would be expected to produce a significant heterogeneous population of blue and colorless plaques (48). Additionally, the presence of uracil in transformed DNA would elicit in vivo BER by the transfected host cells. When M13mp2op14 (U/T) DNA was incubated with U251 whole cell extract and processed, a reversion frequency of 5.2 × 10⁻⁴ was observed after transfecting repaired form I DNA into E. coli NR9162 (mutS) cells. This value was −22-fold greater than that of the control DNA substrate. As expected, this value was reduced (4-fold) when the same repaired form I DNA was transfected into E. coli MC1061 cells capable of removing mismatches from the unmethylated (−)-strand. These results are indicative of nucleotide misincorporation events associated with uracil-initiated base excision repair in human whole cell extracts and provide a measurement of DNA repair synthesis fidelity.

To determine if mismatch repair in the human whole cell extract might influence the determination of reversion frequency, if present, the experiments were repeated using whole cell extracts generated from human LoVo cells. Upon transfection of repaired M13mp2op14 (U/T) DNA into E. coli NR9162, a reversion frequency of 5.4 × 10⁻⁴ was observed, which closely matched that obtained from human U251 whole cell extracts (Table I). Interestingly, similar reversion frequencies were also measured for control M13mp2op14 (A/T) DNA after incubation with U251 or LoVo whole cell extracts and transfection into either E. coli MC1061 or NR9162 cells. Thus, incubation of this substrate with either extract apparently created a background level of mutation that does not differ between U251 and LoVo cells. Together, these results suggest mismatch repair in the human whole cell extracts does not appear to influence the detection of misincorporations that occurred during the BER reaction.

**Mutational Spectrum**—Mutation analysis was performed on individual revertant M13 phage DNA to define the type of misincorporation that occurred during uracil-initiated base excision DNA repair synthesis. Single-stranded DNA was isolated and sequenced from the 78 blue plaques obtained from repaired DNA produced in human U251 whole cell extracts. The distribution of single base substitutions located within the uracil target is shown in Fig. 6. Seventy-four (94%) of the mutations occurred at the first nucleotide of the opal codon, while one mutation (1%) was detected at the second nucleotide position and three (4%) were observed at the third nucleotide position. Thus, almost all of the base substitutions occurred at the location on the uracil target. Interestingly, the major class of these base substitutions was transversion mutations (−94%), and they were nearly equally divided between T to G and T to A changes in the (−)-strand template. Only four mutations were scored as T to C transitions at this site. These results define the uracil-initiated repair-mediated mutation spectrum at this target site.
DNA repair synthesis.

Table I

| DNA          | Plaques scored | Reversion frequency<sup>a</sup> | Plaques scored | Reversion frequency<sup>a</sup> |
|--------------|---------------|-------------------------------|---------------|-------------------------------|
|              | Total         | Blue                          | Total         | Blue                          |
| Control<sup>b</sup> | 216,840       | 17                            | 251,680       | 2                             |
| U251 WCE     | 532,480       | 13                            | 309,218       | 8                             |
| U-T          | 150,705       | 7                             | 150,355       | 19                            |
| LoVo WCE     | 222,820       | 7                             | 48,490        | 2                             |
| A-T          | 121,290       | 66                            | 162,240       | 28                            |

<sup>a</sup> Reversion frequencies were calculated by dividing the number of blue plaques scored by the total number of blue plus colorless plaques. Revertants included dark blue and light blue phenotypes.

<sup>b</sup> The (+) and (−) strand nucleotide at the target site.

<sup>c</sup> Control reaction mixture (500 µl) contained 5 µg of M13mp2op14 (A-T) DNA and substituted whole cell extract dialysis buffer (25 mM Hepes-KOH, 100 mM KCl, 2 mM dithiothreitol, 12 mM MgCl<sub>2</sub>, 1 mM EDTA, and 17% (v/v) glycerol adjusted to pH 7.9) instead of human whole cell extract protein in standard BER reaction mixtures.

**DISCUSSION**

We have examined the ability of human glioblastoma U251 whole cell extracts to conduct uracil-DNA repair using an M13mp2 lacZa DNA-derived substrate containing a site-specific uracil residue. Unlike several recent investigations that have utilized uracil-containing oligonucleotides to characterize BER reactions in eukaryotic cell extracts (15, 16, 18, 49), a covalently closed circular duplex DNA substrate with a defined uracil target was used in this study to facilitate DNA synthesis fidelity measurements and mimic the natural DNA substrate (50). Under the conditions examined, most of the uracil-DNA repair occurred via the BER pathway. Several observations support this conclusion: (i) DNA synthesis was preferentially localized to the *Hin*<sub>F</sub> DNA fragment (529 bp) that contained the uracil target; (ii) repair DNA synthesis within the 529-bp fragment was almost exclusively dependent on the presence of the uracil residue; (iii) DNA synthesis was specifically confined to the (+)-strand DNA that initially contained the uracil target; and (iv) the addition of Ugi to the whole cell extract significantly inhibited repair DNA synthesis. In contrast, these results were not consistent with a mismatch DNA repair pathway, since DNA synthesis was confined to a region significantly <1000 nucleotides, occurred preferentially on only one of the two DNA strands containing the mispair, and efficient repair was observed in LoVo whole cell extracts that are defective in the mismatch repair protein hMSH2 (51, 52).

An examination of the BER reaction kinetics in human U251 whole cell extracts revealed that ~50% of the uracil-DNA substrate was repaired after 60 min of incubation. The ability to achieve this relatively high efficiency of complete repair suggests that a coordinated set of repair reactions occurred without significant accumulation of side products or repair intermediates. This would be consistent with the notion that a concerted BER reaction occurred (37). We also observed that inclusion of Ugi protein in the standard BER reaction reduced the amount of repaired form I DNA from 50 to 3.7%. Thus, approximately 93% of the observed BER appeared to be initiated by a Ugi-sensitive uracil-DNA glycosylase. The small but detectable amount (~7%) of repair that was insensitive to Ugi inhibition suggests that a back-up enzyme or repair pathway exists. A potential candidate for this secondary activity might be the human thymine-DNA glycosylase protein, which is insensitive to Ugi and capable of excising uracil from G-U mispairs (23, 24). While the ability of human thymine-DNA glycosylase to recognize a T-U mispair remains to be established, the enzyme has been shown to exhibit reduced activity toward T-T mispairs (53). Hence, it seems conceivable that human thymine-DNA glycosylase may be capable of removing uracil from a T-U mispair. Another formal possibility would be that glyceraldehyde-3-phosphate dehydrogenase functions as a back-up enzyme. However, the inability of several laboratories to confirm that purified human glyceraldehyde-3-phosphate dehydrogenase possesses significant uracil-DNA glycosylase activity casts doubt on this possibility (6, 14, 30). The biological significance of two uracil-DNA repair systems within human cells remains to be elucidated.

In this study, we present the first fidelity measurements associated with BER DNA synthesis in mammalian whole cell extracts. The analysis utilized a lacZa reversion assay capable of detecting eight out of nine possible base substitutions within the opal (TGA) codon, including all three misinsertions at the uracil target site. Only a misincorporation of dTMP opposite G would be scored as a revertant with a blue plaque phenotype. As expected, most (~95%) of the 78 revertants sequenced following the BER reaction in U251 whole cell extracts contained a base substitution at the first position of the opal codon (nucleotide 78) corresponding to the site of the uracil residue. This was consistent with DNA repair synthesis being initiated at the uracil target on the (+)-strand and proceeding in the 5' to 3' direction away from the opal codon, thus avoiding the second and third nucleotide positions of the codon. We did not attempt to distinguish between BER involving a short or long patch mechanism, since...
the fidelity assay was specifically designed to detect misincorporations only at the uracil target and not downstream (5′ to 3′) along the (−)-strand. Consequently, the error frequencies represent that of short patch BER and the first nucleotide incorporated during long patch BER. Two independent studies recently reported that the short-patch BER provides the dominant pathway for mammalian cells (32, 33). Furthermore, experiments using cell extracts from mouse fibroblasts derived from embryos harboring a homozygous pol β deletion mutation suggested that the rate of short patch BER initiated at AP sites was dramatically reduced but not completely eliminated in the absence of pol β activity (33). In contrast, the rate of long patch BER was apparently unaffected in these pol β-deficient extracts (33). These observations infer that short patch BER is largely pol β-dependent and constitutes the major pathway for repair, whereas DNA polymerase(s) other than pol β are specifically involved in long patch BER. Using the lacZα reversion assay, we observed that uracil-initiated BER in human U251 whole cell extracts occurred with a reversion frequency of 5.2 × 10⁻⁴ (−1 misincorporation/1900 repaired uracil residues). Thus, the BER reaction was considerably more error-prone than the fidelity of DNA synthesis associated with leading or lagging strand DNA replication in human cell extracts (54). The error-prone nature of the BER reaction may necessitate that the repair patch size be kept to a minimum to avoid mutations from being introduced during DNA repair synthesis. Thus, it is not surprising that the repair patch size associated with BER (1–7 nucleotides) is considerably smaller than that associated with nucleotide excision repair (27–29 nucleotides) or mismatch DNA repair (>1000 nucleotides) in human cells (55). The latter two repair pathways do not appear to utilize pol β for the DNA repair synthesis step (55, 56).

A considerable amount of evidence implicates DNA polymerase-β as playing a major role in uracil-initiated BER in mammalian cells (1, 9–12, 18, 33, 36, 37). Recently, the fidelity of in vitro gap filling DNA synthesis by purified pol β has been evaluated (35, 57). Beard et al. (35), using a lacZα opal codon reversion assay to assess the DNA synthesis fidelity on a substrate containing a five-nucleotide gap, demonstrated that purified human pol β produced one revertant per 370 filled gaps (reversion frequency of 27 × 10⁻⁴). This value reflects a somewhat lower fidelity (−5-fold) than that observed for the complete BER reaction in either U251 or LoVo whole cell extracts. This may be explained, in part, by the observation of Chagovetz et al. (57), which indicated that the nucleotide insertion fidelity of purified rat pol β was considerably higher (>10-fold) on a DNA substrate containing a 5′-phosphorylated single nucleotide gap as compared with a gap of six nucleotides. Clearly, other factors such as DNA sequence context and accessory proteins associated with the BER reaction potentially contribute to the observed fidelity of DNA repair synthesis and mutation fixation. In particular, the ability of DNA ligase to act on nicks containing specific 3′-mismatched nucleotides juxtaposed to a 5′-end could significantly influence the mutation spectrum derived from BER. In this regard, recent evidence indicates that bovine DNA ligase III seals a nick containing a 3′-CT mispair with nearly the same efficiency as one containing base-paired termini, whereas, a 3′-GT mispair was recognized with ~5-fold less efficiency (58). A similar preference for 3′-terminal mispairs (3′-CT > 3′-GT) was also observed for bovine DNA ligase I; however, the overall efficiency of DNA joining was reduced 5–10-fold for each individual mispaired termini compared with DNA ligase III (58). These results may provide a partial explanation for the high frequency of C-T versus G-T mispairs detected following the BER assay.

DNA polymerase-mediated base substitutions occur as a con-
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