DNA Base Excision Repair in Human Malaria Parasites Is Predominantly by a Long-Patch Pathway†

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Received October 5, 1999; Revised Manuscript Received November 16, 1999

ABSTRACT: Mammalian cells repair apurinic/apyrimidinic (AP) sites in DNA by two distinct pathways: a polymerase β (pol β)-dependent, short- (one nucleotide) patch base excision repair (BER) pathway, which is the major route, and a PCNA-dependent, long- (several nucleotide) patch BER pathway. The ability of a cell-free lysate prepared from asexual *Plasmodium falciparum* malaria parasites to remove uracil and repair AP sites in a variety of DNA substrates was investigated. We found that the lysate contained uracil DNA glycosylase, AP endonuclease, DNA polymerase, flap endonuclease, and DNA ligase activities. This cell-free lysate effectively repaired a regular or synthetic AP site on a covalently closed circular (ccc) duplex plasmid molecule or a long (382 bp), linear duplex DNA fragment, or a regular or reduced AP site in short (28 bp), duplex oligonucleotides. Repair of the AP sites in the various DNA substrates involved a long-patch BER pathway. This biology is different from mammalian cells, yeast, *Xenopus*, and *Escherichia coli*, which predominantly repair AP sites by a one-nucleotide patch BER pathway. The apparent absence of a short-patch BER pathway in *P. falciparum* may provide opportunities to develop antimalarial chemotherapeutic strategies for selectively damaging the parasites in vivo and will allow the characterization of the long-patch BER pathway without having to knock-out or inactivate a short-patch BER pathway, which is necessary in mammalian cells.

Malaria continues to be an enormous global problem. The lack of an effective vaccine and the development of parasite drug resistance to many of the existing antimalarials threaten to worsen this situation. A better understanding of the parasite's cell and molecular biology may help to produce new targets and strategies for effective control. Two important aspects of parasite metabolism that have received little or no attention are DNA replication and repair, respectively. DNA replication is essential for parasite proliferation and for the maintenance of genome integrity during DNA repair. DNA replication in *Plasmodium falciparum* has only recently been investigated, and three DNA polymerases have been identified in *P. falciparum* (*1*). Only two of these polymerases, DNA polymerases α and δ, have been fully cloned and sequenced (*2*, *3*). Both of these polymerases have homology to yeast and human polymerases, with DNA polymerase δ being more highly conserved than DNA polymerase α (*2*, *3*). The third polymerase, which has only been partially purified in cellular extracts, resembles the mitochondrial DNA polymerase γ of other eukaryotic cells (*4*). To date, there have been no investigations of parasite DNA repair pathways.

DNA repair pathways have been characterized in mammalian cells, yeast, *Xenopus* and *Escherichia coli* (*5*). It is reasonable to assume that all cells and organisms, including *P. falciparum*, have DNA repair activities. Base excision repair (BER)[1] and nucleotide excision repair (NER) are the two major pathways for repairing DNA lesions. Base excision repair is employed on DNA lesions that require precise and rapid attention (*4*, *6*). Substrates for BER include oxidative base damage, multiple forms of alkylation damage, AP (apurinic/apyrimidinic) sites formed by spontaneous loss

† Supported in part by USPHS grant AI41761 to TFT. This investigation received financial support from the UNDP/World bank/WHO Special Program for Research and Training in Tropical Diseases (TDR).

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[1] The abbreviations used are: AP sites, apurinic/apyrimidinic sites; BER, base excision repair; ccc, covalently closed circular; bp, base pair; CHO, Chinese hamster ovary; dRp, deoxyribose phosphate; dRpase, deoxyribophosphodiesterase; EndoIII, *Escherichia coli* Endonuclease III; EndoVIII, *E. coli* Endonuclease VIII; EndoIV, *E. coli* Endonuclease IV; ExoIII, *E. coli* Exonuclease III; FEN1, flap endonuclease I; pol β, DNA polymerase β; pol δ, DNA polymerase δ; pol ϵ, DNA polymerase ϵ; TDG, thymine DNA glycosylase; UDG, uracil DNA glycosylase; UGI, uracil DNA glycosylase inhibitor.

10.1021/bi9923151 CCC: $19.00 © 2000 American Chemical Society
Published on Web 01/04/2000

of bases, and uracil residues in DNA (*4*, *6*). The basic mechanism of BER has been well-conserved, underscoring the physiological importance of the excision repair mode in the cellular response to many of the physiological events or agents that damage DNA. Whereas DNA adducts/lesions repaired by the BER pathway are generally limited to single modifications, bulky lesions (e.g., UV-induced dimers, benzo[*a*]pyrene adducts) are repaired by the NER pathway.

It has been estimated that the spontaneous formation of AP sites in normal cells occurs at the rate of 10 000/cell/day (*7*). This number does not include AP sites formed by DNA glycosylases or free radical induced events. Of the four bases, adenine and guanine are the most susceptible to spontaneous base loss (*8*). Given the unusually high adenine (A) and thymine (T) content of the *P. falciparum* genome (~80%) (*9*) and the spontaneous deadenination of DNA that naturally occurs, it is reasonable to propose that the parasites actively repair AP sites.

Studies in *E. coli* and in human cells have demonstrated that BER of DNA is initiated by DNA glycosylases, which catalyze the hydrolysis of the N-glycosyl bond linking particular damaged bases to the sugar−phosphate backbone. Excision of the damaged bases generates AP sites in the DNA. The phosphodiester bonds 5′ to these sites are incised by an AP endonuclease, generating 3′-OH and 5′-deoxyribose phosphate (dRp) moieties. The latter are removed by a DNA deoxyribophosphodiesterase (dRpase) activity, DNA polymerase fills in the resulting single nucleotide gaps, and the covalent integrity of the DNA strands is restored by a DNA ligase (*10*). DNA polymerase β is apparently the major enzyme that operates during repair synthesis of BER in higher organisms (*11*, *12*), generating a single nucleotide repair patch (*13*, *14*). In the pol β-dependent pathway, pol β catalyzes not only DNA synthesis but also excision of a dRP residue via a β-elimination mechanism (*15*). The pol β-dependent pathway can repair an unmodified natural AP site efficiently, but not a reduced AP site or a synthetic AP site analogue, e.g. 3-hydroxy-2-hydroxymethyltetrahydrofuran, because the latter substrates are not susceptible to β-elimination induced by the 5′-dRpase. The removal and replacement of one nucleotide by the pol β-dependent BER pathway was termed short-patch repair (*16*).

There is evidence of another BER pathway in eukaryotic and prokaryotic cells coined the long-patch BER pathway. This pathway has been demonstrated in *E. coli*, *Xenopus laevis*, and human cells using in vitro systems (*11*, *17*−*19*). In this pathway, the 5′-deoxyribose phosphate moiety is removed as part of a short oligonucleotide by a structure-specific endonuclease, following strand displacement synthesis at the site of incision by an AP endonuclease. This process generates a repair patch comprising more than one nucleotide. In *E. coli*, this structure-specific endonuclease activity may be provided by DNA polymerase I (*17*, *20*), and in mammals, by DNase IV (FEN-1) (*21*). The long-patch BER pathway was PCNA-dependent in mammalian cells, implying a role for DNA pol δ or DNA pol ϵ (*18*). Repair patches up to seven nucleotides were found after the repair of a reduced or oxidized abasic site in oligonucleotides incubated with human cell-free extracts (*16*), or a normal abasic site in closed circular DNA incubated with CHO or HeLa cell-free extracts (*18*). In contrast to the short-patch pathway, the PCNA-dependent pathway was able to repair the synthetic AP site analogue as efficiently as the natural AP site (*19*). However, in both prokaryotes and eukaryotes, the majority of BER is accomplished by a single-nucleotide repair mechanism (*22*, *23*).

We investigated the repair of AP sites in DNA using a parasite cell-free lysate and provide the first evidence for DNA repair activities in *P. falciparum* malaria parasites. We report here that the parasites repair AP sites on DNA predominantly by a long-patch BER pathway. This biology is quite different from mammalian cells, which mainly repair AP sites by a pol β-dependent, one-nucleotide patch BER pathway. The apparent absence of pol β and its associated activities in *P. falciparum* suggests major differences in DNA repair pathways between mammalian cells and *P. falciparum*, which may provide an opportunity to develop antimalarial chemotherapeutic strategies for selectively damaging the parasites in vivo.

MATERIALS AND METHODS

Parasites. The asexual stage of the *P. falciparum* strain 3D7 was maintained in culture in complete medium; RPMI 1640 (Life Technologies,Gibco-BRL, Gaithersburg, MD) supplemented with 25 mM *N*-2-hydroxyethylpiperazine ethanesulfonic acid (HEPES), 24 mM $NaHCO_3$, 21 mM glucose, 2 mM glutathione, 0.44 mM hypoxanthine, 66 mg/L gentamicin, and 10% human serum, at 1% hematocrit, 37 °C under 5% O_2/5% CO_2/90% N_2 and synchronized to ±6−10 h as described previously (*24*, *25*).

Preparation of a Parasite Cell-free Lysate. Late stage parasites (trophozoites and schizonts, 30−40 h postinvasion) were released from the erythrocytes by incubation with an equal volume of 0.15% saponin (Calbiochem, San Diego, CA) in phosphate-buffered saline (PBS), pH 7.5 at 37 °C for 20 min. The intact parasites were separated from the lysed erythrocytes by centrifugation at 5000*g* for 10 min. The parasites were washed 4−5 times in PBS containing a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.3 mg/mL of the following inhibitors; leupeptin, pepstatin, and *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (Boehringer Mannheim, Indianapolis, IN)). The released parasite pellet was then resuspended in 5 pellet vol. of sonication buffer (50 mM Tris, pH 7.6, 10% sucrose, 5 mM EDTA, 10 mM DTT) containing protease inhibitors at the above concentrations. In some instances, 1−2 M NaCl was included in the sonication buffer. The parasites were lysed by sonication for 2 min with a 50% pulse rate and a power setting of 4 using a W-225 Ultrasonic processor (Heat Systems-Ultrasonic, Inc). The sonicate was centrifuged at 32 000 rpm for 1 h at 4 °C in a Ti75 rotor in a Beckman L-80 ultracentrifuge. The resulting supernatant was collected and dialyzed for 16−20 h against lysate buffer (25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 17% glycerol). The protein concentration was determined by the bichoinic acid colorimetric assay from Pierce Biological, Rockford, IL.

Preparation of DNA Substrates for in Vitro Repair. Single-stranded oligonucleotides (28−mer) containing a uracil, cytosine, thymine, or adenine at position 14 and their complementary strands were purchased from Life Technologies Gibco/BRL. Double-stranded oligonucleotides were prepared by mixing equal amounts (mole/mole) of the single-

stranded oligonucleotides and their complimentary strand for 5 min at 75 °C followed by slow cooling to 4 °C.

For the incision assays, the uracil, thymine, or cytosine containing strand (top strand) was labeled on the 5′ end by [γ-^{32}P]-ATP (NEN Life Science, Boston, MA, 3000 Ci/mmol) with T4 polynucleotide kinase (Promega, Madison, WI) prior to annealing. After the sample was annealed, the labeled, double-stranded oligonucleotide was separated from unincorporated nucleotides by purification on Sephadex G25 columns (Boehringer Mannheim). The nucleotide sequences for the (radiolabeled) top strand and the nucleotide at position 14 in the complementary strand (e.g., the designation dU/G means G in the complementary strand is opposite U in the top strand) were as follows:

dU/G5′-TGG CGA AAG GGG G***U***T GTG CTG CAA GGC G-3′

dC/G5′-TGG CGA AAG GGG G***C***T GTG CTG CAA GGC G-3′

dA/T5′-TGG CGA AAG GGG G***A***T GTG CTG CAA GGC G-3′

dT/G5′-TGG CGA AAG GGG G***T***T GTG CTG CAA GGC G-3′

dAP/T5′-TGG CGA AAG GGG G***[AP]***T GTG CTG CAA GGC G-3′

Covalently closed circular (ccc) plasmid DNA containing a single uracil or a synthetic AP site analogue (3-hydroxy-2-hydroxymethyltetrahydrofuran) was prepared as previously described (*19*). In prelabeled cccDNA, ^{32}P was incorporated several nucleotides 5′ or 3′ from the lesion. Linear DNA was prepared by digestion of the cccDNA with *Pvu*II (Promega), which generated a labeled 382-base pair fragment carrying the AP site in the middle of the fragment and an unlabeled 2.8 kilobase fragment without an AP site. Double-stranded closed circular M13 DNA containing a single uracil was constructed as described (*22*), by priming single-stranded M13 DNA with the oligonucleotide 5′-pATATACCGCG-G***U***CGGCCGATCAAGCTTATT-3′.

Incision Assays with a P. falciparum Cell-free Lysate. Standard in vitro incision assays were performed in a 20 μL reaction mixture containing 0.1 pmol of the 5′ end-labeled oligonucleotide, 70 mM HEPES, pH 7.8, 1 mM EDTA, 1 mM βME, and 10−20 μg of cell-free lysate protein. To visualize the UDG glycosylase activity, the oligonucleotides were posttreated with heat and alkali to induce β/δ-elimination and strand breakage. For analysis of AP endonuclease activity, DNA was incubated in buffer containing 200 mM KCl. Reactions were carried out at 37 °C for 30 min and then posttreated as described in the text. After posttreatment, 100 μL of formamide loading buffer (47.5% formamide, 10 mM EDTA, 0.025% Bromophenol Blue, 0.025% Xylene Cyanol FF) was added and a 4 μL aliquot was loaded onto a 15% denaturing polyacrylamide gel for analysis by electrophoresis. Incision products were detected by autoradiography of the wet gels.

Base Excision Repair by a P. falciparum Cell-free Lysate. For analysis of repair synthesis on duplex oligonucleotides, standard in vitro repair reactions were performed as described previously (*26*), with slight modifications. Standard reaction mixtures (20 μL) contained 10−20 pmol of the 28-mer duplex DNA, 50 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 1.5 mM β-mercaptoethanol (βME), 3 mM ATP, 1 μCi [α-^{32}P]-dATP (3000 Ci/mmol), 10 μM each of dCTP, dGTP, and dTTP, 30 mM creatine phosphate, 0.12 μg creatine kinase, 0.6X polymerase buffer (30 mM Tris-HCl, pH 8.8, 6 mM KCl, 6 mM $MgCl_2$, 0.24 mg/mL BSA, 0.6 mM DTT, 0.9% glycerol) from Trevigen, (Gaithersburg,MD) and 10−20 μg of cell-free lysate protein. Repair reactions were performed at 37 °C for the desired time and stopped by the addition of SDS to a final concentration of 0.6%. Samples were treated with 0.06 mg/mL proteinase K for 1 h at 37 °C; 0.013 mg/mL tRNA (5′-3′, Boulder, CO) was added as a carrier. Reactions were stopped by the addition of 150 μL PK-stop (10 mM Tris-HCl, pH 7.5, 300 mM Na-acetate, 10 mM EDTA, 0.5% SDS), and DNA was extracted by phenol-chloroform and precipitated with ethanol. Repair reactions separated by electrophoresis on 15% denaturing polyacrylamide gels as indicated. Repair synthesis was visualized by autoradiography of wet gels.

Repair synthesis on the cccDNA was carried out under similar reaction conditions as for the oligonucleotides. A series of 20 μL reactions containing 10 ng of cccDNA supplemented with 1000−5000 cpm of radiolabeled cccDNA were stopped by the addition of SDS to a final concentration of 0.6%. Samples were treated with 0.06 mg/mL proteinase K for 1 h at 37 °C; 0.013 mg/mL tRNA was added as a carrier. The reaction was stopped by the addition of 150 μL PK-stop (10 mM Tris-HCl, pH 7.5, 300 mM Na-acetate, 10 mM EDTA, 0.5% SDS), and DNA was extracted by phenol and chloroform and precipitated with ethanol. After ethanol precipitation, samples were digested with *Hinf I* (New England Biolabs, Beverly, MA) or *PvuII* and subjected to electrophoresis on denaturing polyacrylamide gels (20% for *HinfI* and 6% for *Pvu II* digests).

DNA repair synthesis reactions with closed circular M13 DNA containing a single uracil were carried out at 32 °C for 2 h as described (*22*). After the reaction, DNA was purified from the reaction mixture by phenol/chloroform extraction and the samples digested with HindIII or HpaII for 3 h at 37 °C in a buffer containing 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, and 20 U of each indicated restriction endonuclease. Restriction products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel. The amount of radioactivity in individual bands was quantified with a PhosphorImager (Molecular Dynamics).

Additional Enzymes and Reagents. Uracil DNA-glycosylase (UDG) was purchased from Epicentre Technologies, Madison, WI. Uracil DNA-glycosylase inhibitor (UGI) was from New England Biolabs. E. *coli* Endonuclease IV and human DNA polymerase β were from Trevigen. Proteinase K was from Boehringer Mannheim (Indianapolis, IN). Deoxyribonucleotides were from Amersham. ATP, creatine kinase, and phosphocreatine were from Sigma (St. Louis, MO). Recombinant human DNA ligase I (*27*) was a gift from Alan Tomkinson.

RESULTS

Identification of UDG Activity in the P. falciparum Cell-free Lysate. Base excision repair of DNA lesions is initiated

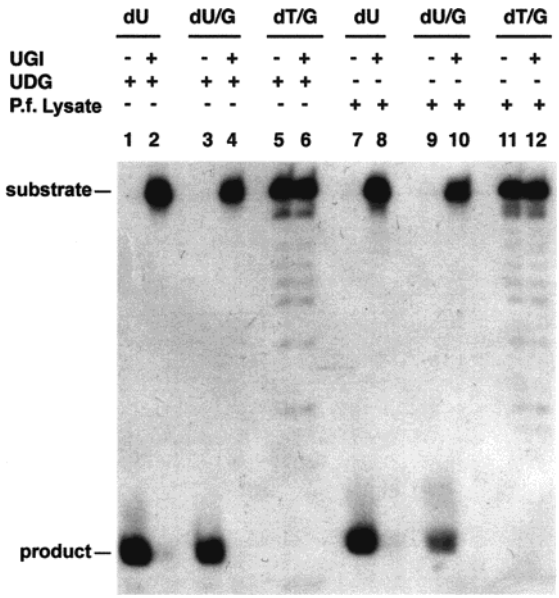

FIGURE 1: A *P. falciparum* cell-free lysate removes uracil from DNA by a uracil- DNA glycosylase (UDG) activity. Incision assays were performed on a 5′-end ^{32}P-labeled, 28-mer, single-stranded oligonucleotide containing uracil at position 14 from the 5′-end (lanes 1, 2, 7, 8), or 5′-end ^{32}P-labeled, 28-mer, duplex oligonucleotides containing uracil (lanes 3, 4, 9, 10) or thymidine (lanes 5, 6, 11, 12) at position 14 from the 5′-end. Oligonucleotides were incubated with 0.1 U of *E. coli* UDG (lanes 1−6) or 15 μg of cell-free lysate protein (lanes 7−12) in the presence or absence of a specific UDG inhibitor (UGI) for 30 min at 37 °C. Reactions were subsequently posttreated with 0.2 M NaOH for 30 min to cleave the oligonucleotide at the site of glycosylase activity. Samples were directly loaded onto 15%, 8.3 M urea polyacrylamide gels. No activity was detected in lysates from uninfected erythrocytes.

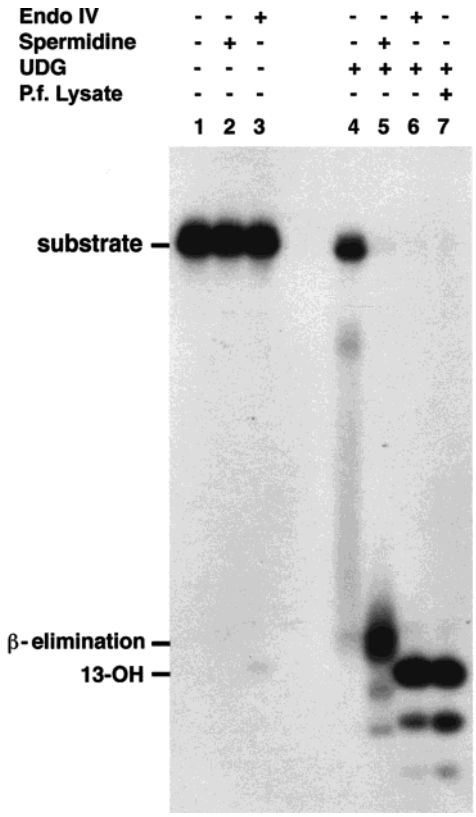

FIGURE 2: The *P. falciparum* cell-free lysate contains AP endonuclease activity. Oligonucleotides were either untreated (lanes 1, 4), treated with 1 mM spermidine (lanes 2, 5), 5 U *E. coli* endonuclease IV (lanes 3, 6) or 15 μg of cell-free lysate proteins (lane 7) for 30 min at 37 °C. Reactions were directly loaded onto a 15% 8.3 M urea polyacrylamide gel. The positions of the oligonucleotide markers (endonuclease markers) are indicated on the left. No activity was detected in lysates from uninfected erythrocytes.

by DNA glycosylases. In mammalian cell systems, two distinct glycosylases exist that are capable of removing uracil from DNA. These differ in their substrate requirements and their sensitivity to the specific UDG inhibitor, UGI. The major glycosylase responsible for the removal of uracil from DNA is uracil-DNA glycosylase (UDG), which is capable of removing uracil from single- and double-stranded DNA. It is unable to remove thymine from DNA containing a T/G mismatch and is completely inhibited by UGI. Thymine-DNA glycosylase (TDG) or mismatch DNA glycosylase (MDG) also removes uracil from DNA. This glycosylase primarily removes thymine from T/G mismatches; however, TDG can also efficiently remove uracil from U/G mismatches in double-stranded DNA. Additionally, TDG cannot remove uracil from ssDNA and is insensitive to the inhibitor UGI.

To determine if *P. falciparum* malaria parasites contained a UDG activity, the ability of the parasite cell-free lysate to remove a single uracil or thymine 14 bases from the 5′ end of ^{32}P 5′-end labeled oligonucleotides (28-mer) was investigated. As seen in Figure 1, the cell-free lysate removed uracil from single- and double-stranded oligonucleotides (Figure 1, lanes 7−10). The cell-free lysate did not remove thymine from T/G mismatches (Figure 1, lanes 11, 12) and was inhibited by UGI (Figure 1, lanes 8, 10). As a control, the same conditions were run in parallel using *E. coli* UDG (Figure 1, lanes 1−6). Comparison of the activity of the parasite lysate to *E. coli* UDG revealed that the cell-free lysate possessed a uracil-DNA glycosylase activity.

Identification of AP Endonuclease Activity in the Parasite Lysate. Newly formed AP sites in DNA are incised by an AP endonuclease in the BER pathway. In prokaryotic cells such as *E. coli*, two classes of AP endonuclease function to process abasic sites. Class I AP endonucleases, such as Endonuclease III (EndoIII), and Endonuclease VIII (EndoVIII) have an associated AP lyase activity and cleave AP sites on the 3′-side by β-elimination, generating an α,β-unsaturated aldehyde (*28*). Class II AP endonucleases such as Exonuclease III (ExoIII) and Endonuclease IV (EndoIV) from *E. coli* cleave abasic sites on the 5′-side, generating 3′-OH and 5′-deoxyribosephosphate moieties (*28*). To determine if the parasite lysate possessed AP endonuclease activity, oligonucleotides were ^{32}P-labeled on the 5′-end and treated with UDG to generate an AP site. The AP site containing oligonucleotide, dAP/G, was incubated with the cell-free lysate. As seen in Figure 2, treatment of the control, uracil-containing oligonucleotide with 1 mM spermidine (which breaks the DNA at the AP site by a β-elimination reaction) or 5 U of *E. coli* AP EndoIV, were without effect on oligonucleotides lacking an abasic site (Figure 2, lanes 1−3). When the uracil was removed to generate an AP site, the oligonucleotide was incised by treatment with 1 mM spermidine or EndoIV, indicating the presence of abasic sites (Figure 2, lanes 5, 6). As a control for the stability of the AP site during electrophoresis, dAP/G treated only with UDG was also analyzed without posttreatment (lane 4) and found to remain largely intact (Figure 2, lane 4). Spermidine cleaved the AP site formed by UDG treatment by β-elimina-

tion on the 3′-side of the site, resulting in a 13 nucleotide fragment containing an α,β-unsaturated aldehyde on the 3′-end (Figure 2, lane 5). Endonuclease IV cleaved the AP site on the 5′-side, resulting in a 13 nucleotide fragment with a 3′-OH moiety (Figure 2, lane 6). The fragments generated by spermidine and Endonuclease IV were compared to a 13-mer oligonucleotide containing a 3′-OH. As expected, the fragment resulting from EndoIV treatment migrated at the same position as the 13-mer with a 3′-OH moiety (data not shown.) Incubation of dAP/G with the parasite lysate revealed incision of the oligonucleotide, releasing a 13-nucleotide fragment that migrated at the same position as that of dAP/G treated with AP EndoIV (Figure 2, compare lane 7 with 6). The cell-free lysate was also capable of processing dU/G into the same 13-mer fragment, indicating the concerted actions of a UDG activity followed by AP endonuclease activity (data not shown).

DNA Base Excision Repair of an AP Site in Short Duplex Oligonucleotides. Following base removal and incision of the AP site, the subsequent events in the DNA repair process involve two branching pathways: a pol β-dependent, short-patch BER pathway, or a PCNA-dependent, long-patch repair pathway. Dianov et al. (*23*) reported that a regular AP site in a duplex oligonucleotide was corrected by the incorporation of a single nucleotide, whereas repair patches of two to six nucleotides were found after repair of a reduced AP site (*16*). Human AP endonuclease, DNA pol β, and DNA ligase I or III were sufficient to repair the regular AP site. The structure-specific nuclease DNase IV (FEN1) was essential for repair of the reduced AP site, which occurred through a long-patch BER pathway. The long-patch BER pathway was largely dependent on DNA pol β in cell extracts, but the reaction could be reconstituted with either DNA pol β or δ (*16*).

To determine if the parasite lysate contained activities necessary for completion of the BER pathway, i.e., DNA repair synthesis and ligation, repair synthesis was measured by monitoring the incorporation of [α-^{32}P]-dAMP into a double-stranded 28-mer oligonucleotide containing a single, natural- (dAP/T) or $NaBH_4$-reduced- ($dAP_{reduced}$/T) AP site. Figure 3 shows complete DNA repair of both substrates by a long-patch BER pathway, with similar kinetics for repair synthesis (patches up to nine nucleotides) and ligation (28-mer). The repair of the regular AP site by a long-patch BER pathway was unexpected, given previous results obtained with mammalian cells (*16*, *23*, *29*), wherein repair of this type of lesion on a short duplex oligonucleotide was by the short-patch, pol β-dependent BER pathway. Furthermore, while the reduced AP site was repaired quicker and more efficiently than the regular AP site in ref *16*, the kinetics of BER of either substrate by the *P. falciparum* cell-free lysate were very similar (Figure 3, compare A and B). The longest repair patch was five nucleotides for the reduced AP site in the reconstituted human BER system (*16*), whereas we observed up to nine nucleotide repair patches with the parasite lysate. Although long-patch BER appeared to be the predominant pathway, a contribution from a one nucleotide, short-patch repair pathway could not be excluded.

The fully repaired product in Figure 3, A and B, was a minor product, which we suspected might be due to a weak DNA ligase activity in the lysate. The addition of recombinant human DNA ligase I to the parasite lysate greatly

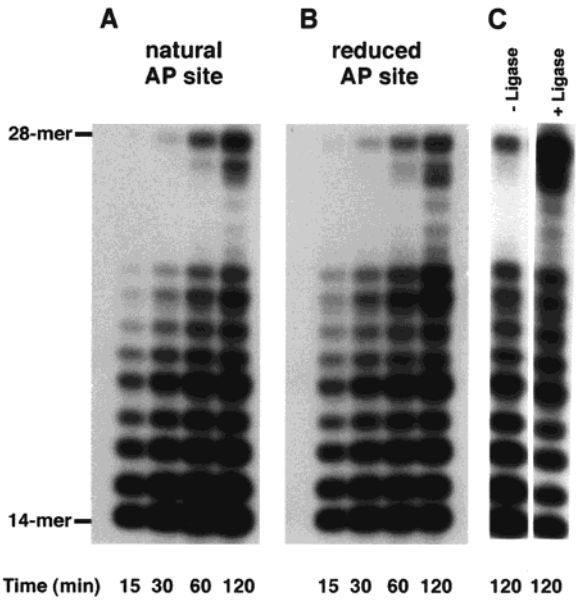

FIGURE 3: Regular or reduced AP sites on duplex oligonucledtides are repaired by a long-patch BER pathway by the *P. falciparum* cell-free lysate. Repair reactions containing a duplex oligonucleotide with a (A) natural (dAP/T) or (B) a $NaBH_4$-reduced ($dAP_{reduced}$/T) AP site were incubated for 15−120 min with 15 μg of cell-free lysate protein in a mixture containing 1 μCi [α-^{32}P]-dATP and 10 μM dCTP, dGTP, and dTTP. (C) dAP/T incubated for 120 min with 15 μg of cell-free lysate protein in a mixture containing 1 μCi [α-^{32}P]-dATP and 10 μM dCTP, dGTP, and dTTP in the absence and presence of 10 pM human DNA ligase I. Samples were analyzed on 15% gels. Repair synthesis was measured by incorporation of [α-^{32}P]-dATP into the oligonucleotide as visualized by autoradiography. No activity was detected in lysates from uninfected erythrocytes.

stimulated the formation of the fully repaired product (Figure 3C).

Long-patch Repair of a Covalently Closed Circular Plasmid DNA Containing a Single AP Site. The use of the covalently closed circular (ccc) DNA substrate described in Matsumoto et al. (*19*), which was ^{32}P-labeled 5′ or 3′ of the AP site (Figure 4A), enables the analysis of intermediates and fully repaired products in the BER pathway. Recent observations in mammalian systems indicated that the long-patch, PCNA-dependent pathway for BER was fully functional on circular, but not linear DNA, while the short-patch, pol β-dependent pathway repaired both circular and linear DNA (*30*). The results in Figure 4 revealed that DNA repair of the circular DNA, containing a natural- (Figure 4B) or synthetic-AP site (Figure 4C), occurred at similar rates and was characteristic of repair by a long-patch BER pathway. DNA repair synthesis filling gaps up to nine nucleotides and strand ligation were observed after 30−60 min of incubation with the parasite lysate. As was observed with the short, double-stranded oligonucleotides, the addition of human DNA ligase I to the parasite lysate greatly stimulated the formation of fully repaired product from 15% to ∼80% after a 120 min incubation (data not shown). While repair of the substrate containing the synthetic AP occurred by a long-patch pathway, a contribution from a short-patch BER pathway to the repair of the substrate bearing the natural AP site could not be excluded.

An AP Site in a Long, Linear DNA Fragment is Repaired by a Long-patch BER Pathway. To further investigate the apparent differences in DNA repair pathways between

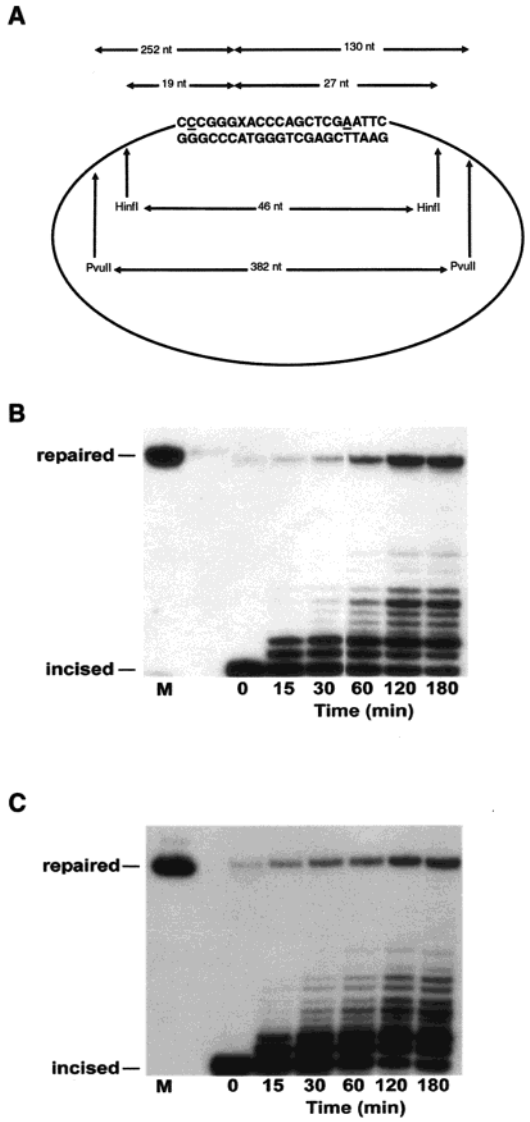

FIGURE 4: A natural or synthetic AP site in cccDNA is repaired by a long-patch BER pathway by the *P. falciparum* cell-free lysate. Repair reactions utilizing (A) cccDNA, labeled on the 5′-side of the AP site (at C in the top strand), containing a (B) natural- or (C) synthetic- AP site (at position X in the top strand), were incubated for 0−180 min with 15 μg of cell-free lysate protein. Prior to adding the lysate, the DNA was incubated with 1 U of *E. coli* UDG and 5 U of *E. coli* Endo IV for 30 min at 37 °C to generate the natural, preincised AP site; the preincised AP site in the DNA with the synthetic AP site was produced by incubating the DNA with 5 U of *E. coli* Endo IV for 30 min at 37 °C. After incubation in the absence or presence of lysate proteins for the desired time, the DNA was recovered and subjected to restriction digestion with 2 U of HinfI to release a fragment containing the AP site. The DNA was loaded onto 20%, 8.3 M urea polyacrylamide gels for electrophoresis. Products were visualized by autoradiography. DNA marker for an AP endonuclease repaired (M) product is indicated on the left.

malaria parasites and mammalian cells, we compared the repair of a synthetic AP site in the cccDNA and a linear 382 base pair PvuII fragment excised from the cccDNA. The *P. falciparum* cell-free lysate repaired the synthetic AP site on both the circular DNA and the linear fragment by a long-patch repair pathway (Figure 5, compare lanes 2 and 3). The kinetics of DNA synthesis and ligation were very similar for the two substrates, with 12% of the circular DNA and 8% of the linear DNA completely repaired after 3 h of

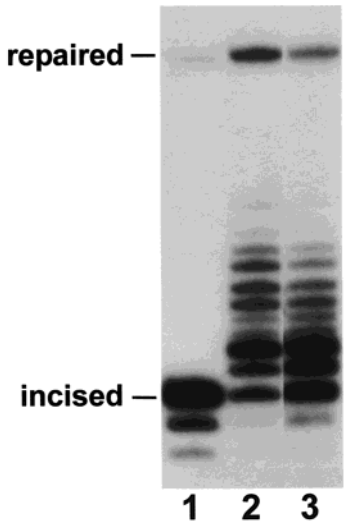

FIGURE 5: AP sites in linear, duplex DNA and cccDNA are repaired by a long-patch BER pathway. Repair reactions containing the cccDNA (lanes 1, 2) with a synthetic AP site shown in Figure 4A or a 382 nucleotide linear fragment of the cccDNA (lane 3) with a label on the 5′-side of the AP site (at C in the top strand), were incubated for 0 min (lane 1) or 180 min (lane 2, 3) with 15 μg of cell-free lysate protein. Prior to incubation with the lysate, the cccDNA was preincised as described in Figure 4. The linear DNA was preincised by cutting the cccDNA with PvuII, followed by inactivation of the enzyme by heating at 75 °C for 5 min. This DNA was then incubated with Endo IV as described in Figure 4. After incubation of the DNA with the lysate, the DNA was recovered and subjected to restriction digestion with 2 U of HinfI to release a fragment containing the AP site. The DNA was loaded onto 20%, 8.3 M urea polyacrylamide gels for electrophoresis. DNA markers for AP endonuclease incised and repaired products are indicated on the left. Products were visualized by autoradiography and analyzed by phosphorimaging.

incubation with the parasite lysate (lanes 2 and 3). Thus, regular or synthetic AP sites on short or long oligonucleotides and circular DNA were repaired by the parasite lysate by a long-patch BER pathway.

Base Excision Repair by P. falciparum is Predominantly by a Long-patch Pathway. While the results in Figures 3−5 suggested that the repair of AP sites in a variety of DNA substrates was predominantly by a long-patch BER pathway, the contribution to the repair of regular AP sites by a short-patch pathway was difficult to assess. To address this issue, we measured the size of the repair patch produced in human and parasite cell extracts using a double-stranded DNA construct containing a single uracil−guanine base pair (Figure 6A). The nucleotide sequence around the uracil residue was designed such that the restriction endonuclease HpaII would cleave one base 3′ to the lesion (Figure 6A; (*22*)). Thus, the gap filling of a single nucleotide at the site of the lesion would be detected as the presence of label only in the 49 bp HindIII−HpaII fragment, whereas further elongation of the repair patch would result in the additional labeling of the 10 bp HpaII−HindIII fragment. DNA was incubated with the different cell extracts in the presence of [α-^{32}P]dCTP, purified, and subjected to restriction analysis. Radiolabeled dCTP was used, since it could be incorporated at positions 1, 4, 5, or 9 in the 10 bp HpaII−HindIII fragment. The incorporation of radionucleotide into the 59 bp HindIII fragment was highly DNA damage-specific, as there was no detectable incorporation observed with the control plasmid (data not shown). The analysis of the repair

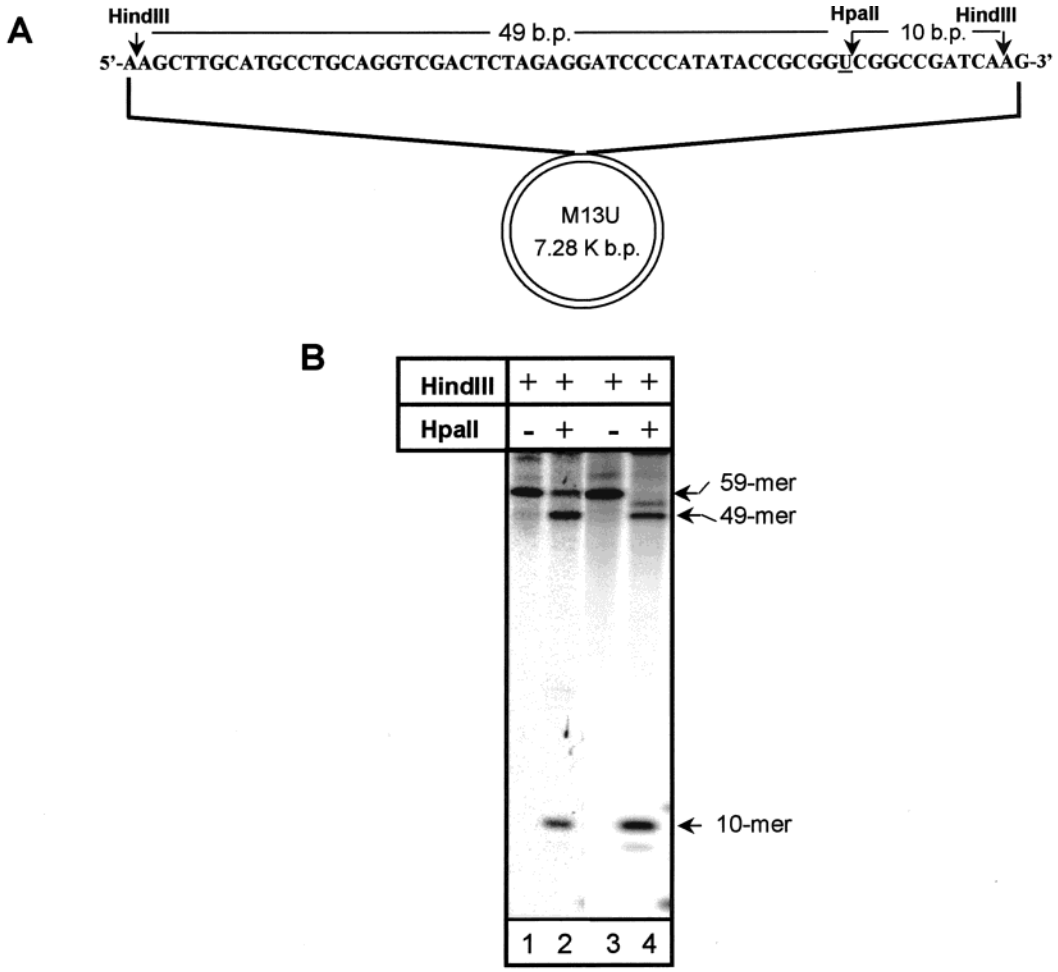

FIGURE 6: Restriction analysis of a single-uracil DNA construct repaired by a human or *P. falciparum* cell lysate. (A) Nucleotide sequences and restriction sites in the vicinity of uracil (*U* in the top strand). (B) Substrate DNA was incubated with 100 μg of whole cell extract protein from the human lymphoblastoid cell line AG9387 (lanes 1 and 2) or parasite cell lysate (lanes 3 and 4) in the presence of [α-^{32}P]-dCTP under the conditions described in Materials and Methods. The DNA was recovered from the reaction mixture, digested with restriction enzymes, and analyzed by electrophoresis on a 20% polyacrylamide gel.

gap size revealed that the human cell extract predominantly repaired uracil via the single-nucleotide patch pathway, since about 70% of the dCMP was incorporated into the 49-mer HindIII−HpaII fragment (Figure 6B, lane 2). This result was in good agreement with a previous investigation, which utilized a human cell extract to examine BER in a similar DNA substrate (*22*). By contrast, in the parasite lysate, the repair gap was at least 4−5 nucleotides long, as up to 70% of incorporated dCMP was found in the 10-mer HpaII−HindIII fragment (Figure 6B, lane 4). These results demonstrated that the long-patch repair pathway was responsible for the repair of uracil in parasite cell lysate.

Evidence for a Flap Endonuclease Activity in the Parasite Long-patch BER Pathway. The use of the cccDNA-labeled 3′ of the AP site (Figure 4A) permits the analysis of the excision of the nucleotides in a 5′ → 3′ direction from the AP site. Comparison of the activity of the lysate on the cccDNA and the PvuII fragment revealed a pattern (Figure 7A), which was the mirror image of the long-patch repair observed for the same substrates in Figure 5. The results suggested the presence of a flap endonuclease activity in the parasite lysate, which excised 2−9 nucleotide patches from the flap created by DNA repair synthesis. To examine this activity in more detail, a kinetic analysis of the 3′-labeled cccDNA was undertaken (Figure 7B) and the repaired products compared to those obtained with the 5′-labeled cccDNA in Figure 4. The excision pattern, which closely mirrored the DNA repair synthesis pattern, suggested that the parasite long-patch BER pathway utilized a flap endonuclease activity to remove the displaced single-stranded DNA flap that formed during repair, similar to FEN1 or DNAse IV used in the long-patch BER pathway in mammalian cells.

DISCUSSION

DNA repair is essential for cell survival and has been studied extensively in a variety of eukaryotic and prokaryotic systems. In protozoa, such as *Plasmodium*, DNA repair has not been investigated. An understanding of the biochemical processes involved in DNA replication and repair of this parasite will produce valuable information for the design of therapies against this important pathogen. In this investigation, we provided the first evidence for DNA repair activities in a parasitic organism. The removal of uracil from single- or double-stranded DNA by the parasite lysate was by a uracil-DNA glycosylase activity. A parasite AP endonuclease activity similar to the *E. coli* class II AP endonuclease, EndoIV, or the yeast homologue APN1 was also identified.

In mammalian cells, regular AP sites in DNA are predominantly repaired by the short-patch BER pathway; a

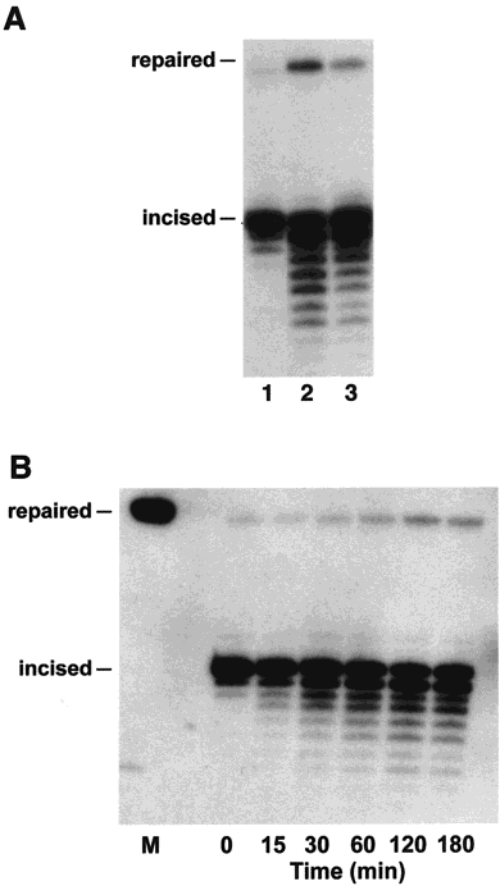

FIGURE 7: Evidence for a flap endonuclease activity in the *P. falciparum* cell-free lysate. Repair reactions containing (A) cccDNA (lanes 1, 2) or a linear fragment (lane 3) with a label on the 3′-side of the AP site (A in the top strand of Figure 4A) were incubated for 0 min (lane 1) or 180 min (lane 2, 3) with 15 μg of cell-free lysate protein. (B) Repair reactions containing cccDNA with a label on the 3′-side of the AP site were incubated for 0−180 min with 15 μg of cell-free lysate proteins. After incubation, the DNA was recovered and subjected to restriction digestion with 2 U of HinfI to release a fragment containing the AP site. The DNA was loaded onto 20%, 8.3 M urea polyacrylamide gels for electrophoresis. Products were visualized by autoradiography. DNA markers (M) for incised and fully repaired products are indicated on the left.

small proportion of such sites may be repaired by the long-patch pathway (*23*). By contrast, oxidized and reduced AP sites are refractory to repair by the short-patch pathway and can only be processed by the long-patch pathway (*16*). Our results suggested that the P. *falciparum* cell-free lysate repaired regular and synthetic AP sites in various DNA substrates by a long-patch BER pathway. To determine the contributions of short-patch and long-patch BER for processing AP sites in DNA, we examined the involvement of different repair pathways in the processing of uracil in human and parasite cell extracts using restriction analysis of in vitro repaired DNA. This method allowed us to analyze the final products of repair events. We found that both human and parasite cell extracts efficiently removed uracil from DNA and incorporated dCMP into repair patches within a 59-mer HindIII−HindIII restriction fragment (Figure 6, lanes 1 and 3). Upon analysis of the distribution of radioactivity within this fragment, we found that human cell extracts repaired uracil in DNA preferentially by a single nucleotide replacement mechanism and incorporated 70% of radioactivity into a 48-mer HindIII−HpaII fragment (Figure 6, lane 2). However, some repair takes place by a long-patch repair mechanism resulting in labeling of 10-mer HpaII−HindIII fragment as shown previously (*14*, *23*). In contrast, analysis of the repair incorporation supported by the parasite lysate indicated that 70% of the radioactivity was incorporated into a 10-mer HpaII−HindIII fragment and 30% of radioactivity was found in the 49-mer HindIII−HpaII restriction fragment. The longest patch size detected during repair by the parasite lysate was nine nucleotides (as seen in Figures 3−5, 7). If all of the BER was by a long-patch pathway and the average patch size was nine nt, then the theoretical distribution of radioactivity incorporation into the 49-mer HindIII−HpaII (containing one cytosine) and 10-mer HpaII−HindIII fragments (containing an additional three cytosines in a gap) would be 25 and 75%, respectively. These values are in a close agreement with our observed distribution of radioactivity between the fragments (30 and 70%). Since filling at the first cytosine in the HindIII−HpaII 49-mer would occur in long-patch repair, the radioactivity in this fragment can never be less that 25%, even if the repair is exclusively by long-patch repair. These data clearly indicate that in the parasite lysate, the long-patch mechanism is the primary pathway for the repair of uracil in DNA.

A major difference exists between BER pathways in *P. falciparum* and their mammalian host. Mammalian cells repair the majority of AP sites in DNA by a pol β-dependent branch of the BER pathway (*12*, *14*, *23*), whereas *P. falciparum* does not appear to utilize this pathway. At present, there is no evidence of a DNA polymerase β homologue in *P. falciparum*; however, the malaria genome project is incomplete with only some of the 14 chromosomes having been completely sequenced. Activity gels revealed the presence of DNA polymerase α, but not β, in *P. falciparum* parasites (*31*). We probed the parasite lysate and the human cell extract used in Figure 6 with an anti-human DNA pol β antibody; a band at the reported molecular weight of human DNA pol β was observed in the human cell extract, whereas no reactivity was observed in the parasite lysate (data not shown).

It is possible that a *P. falciparum* pol β could be inactivated in the preparation or storage of the parasite lysate or that the incubation conditions of the repair synthesis reactions do not support DNA pol β activity. The latter possibility is unlikely, since the addition of human DNA pol β to a nucleotide-depleted lysate containing an oligonucleotide with a regular AP site resulted in the stimulation of DNA repair synthesis by a one-patch repair mechanism (data not shown). It was reported that DNA pol β tightly associates with chromatin, and high salt was necessary to extract the enzyme (*32*, *33*). To determine if the use of a low salt lysate buffer artificially excluded a parasite DNA pol β, the parasite lysate was also prepared with sonication buffer containing 1−2 M NaCl. Essentially identical results were obtained with either lysate preparation (data not shown). Taken together, our results suggest the absence of a DNA pol β in *P. falciparum*.

Simple BER is the predominant type of BER used by human cells and mouse fibroblasts, but apparently not *P. falciparum*. *P. falciparum* may share some similarity with yeast in that the BER in the latter does not utilize pol β, where the process is carried out by a polymerase δ or ϵ, but differ in that AP sites are predominantly repaired by a one nt patch BER pathway (*34*). Our results indicated that *P.*

falciparum repaired AP sites in DNA by a long-patch pathway that had some similarities, and a few differences, with the mammalian PCNA-dependent, pol δ or ϵ mediated pathway. A *P. falciparum* pol δ gene has been identified and sequenced (*2*), but there is no evidence of a pol ϵ gene. One major difference is that the *P. falciparum* lysate repairs regular or reduced AP sites on linear DNA substrates by a long-patch pathway, whereas the PCNA-dependent long-patch BER pathway in mammalian cells is nonfunctional on linear substrates. Proliferating cell nuclear antigen (PCNA) plays an essential role in DNA replication and repair. It is a protein of approximately 29 kDa that forms a homotrimer with a torus structure. Double-stranded DNA can pass through the inside cavity of the PCNA trimer. Loading of PCNA onto DNA is facilitated by the replication factor C (RF-C), resulting in a PCNA/RF-C clamp (*35*). The formation of the PCNA clamp is a prerequisite to efficient DNA synthesis by pol δ or pol ϵ. Investigations demonstrating the PCNA-dependent, long-patch BER pathway have utilized circular DNA substrates. Podust et al. (*36*) reported that PCNA could be loaded more stably on circular DNA than on linear DNA in vitro. Unstable loading of the PCNA onto linear DNA is due to falling off of the PCNA clamp from linear ends after sliding along it. This may be why linear DNA substrates do not support the PCNA-dependent, long-patch pathway. This suggests that either the *P. falciparum* PCNA is structurally different from mammalian PCNA, which is possible given its low sequence homology with PCNA from other species (*37*), or that the *P. falciparum* long-patch pathway is not PCNA dependent, making it a unique DNA repair pathway. An anti-human PCNA antibody was nonreactive with the parasite lysate by Western blotting, and the addition of a (mammalian)PCNA neutralizing antibody to the parasite lysate did not alter the long-patch repair of DNA containing an AP site (data not shown). These results could suggest that the *P. falciparum* long-patch repair pathway does not require PCNA, or that the parasite PCNA is sufficiently different from the mammalian PCNA not to be recognized by the antibody. If the parasite PCNA is structurally (and functionally) different from human PCNA, then it might make an excellent target for antimalarial chemotherapy given its role in both DNA replication and repair.

The results in Figure 7 suggested the involvement of a flap endonuclease activity in the *P. falciparum* long-patch BER pathway. Examination of the timecourse of products formed with the 3′- and 5′-labeled cccDNA in Figures 7 and 4, respectively, revealed essentially mirror images. This is consistent with the synthesis of repair patches up to nine nucleotides and the simultaneous cleavage of the resulting flap structures. The identification and characterization of a parasite flap endonuclease could be helpful in the elucidation of parasite DNA replication and repair pathways and could provide insight into the origin of the unusual A−T richness (>80%) of the *P. falciparum* genome.

DNA joining events are required for the completion of DNA replication and repair. The sealing of DNA breaks generated during BER is done by DNA ligases (*38*). PCNA dependent long-patch BER repair requires DNA ligase I or III, whereas pol β-dependent, short-patch repair requires DNA ligase III (*39*). The results of the repair synthesis reactions in Figures 3−7 suggested the presence of DNA ligase activity in the *P. falciparum* lysate. While a DNA ligase gene has not yet been identified in *P. falciparum*, the C-terminal amino acid sequences DSXDLXVXGAYYGKG and GXSXRFPREFXRIREDK are highly conserved among DNA ligase I from several different species (*40*), including the trypanosomatid *Crithidia fasciculata* (*41*). Thus, it is reasonable to propose the presence of a functional homologue in *P. falciparum*.

To summarize, our investigation revealed that *P. falciparum* repaired AP sites and uracil residues in DNA predominantly by a long-patch BER pathway. This biology is different than mammalian cells, which predominantly repair AP sites by a DNA polymerase β-dependent, one-nucleotide patch BER pathway. The apparent absence of DNA pol β and its associated activities in *P. falciparum* suggests major differences in DNA repair pathways between mammalian cells and *P. falciparum*. It is tempting to speculate that given the evolutionary pressure placed on *P. falciparum* the parasite may be considered to be more evolved than mammalian cells or *E. coli* with respect to BER, and that long-patch BER is a more modern DNA repair pathway than short-patch BER. Furthermore, considering the A−T richness of the *P. falciparum* genome, long-patch BER may be more efficient for repairing multiple errors, which occur in close proximity in the parasite DNA, than a short-patch BER pathway. Characterization of DNA repair pathways will provide important, new information about parasite biology, and the opportunity to exploit differences in DNA repair between *P. falciparum* and mammalian cells to develop strategies for selectively damaging the parasites in vivo. Furthermore, the *P. falciparum* system provides the opportunity to investigate the long-patch BER pathway without the need to inactivate or knockout the short-patch pathway that predominates in all other systems investigated to date.

ACKNOWLEDGMENT

We thank Allison McKenna for preparing the cccDNA, Alan Tomkinson for the gift of human DNA ligase I, and Brian Kilbey for helpful discussions.

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BI9923151