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Malaria parasites utilize both homologous recombination and alternative end joining pathways to maintain genome integrity

Laura A. Kirkman[1], Elizabeth A. Lawrence[1] and Kirk W. Deitsch[2,*]

[1]Department of Internal Medicine, Division of Infectious Diseases and [2]Department of Microbiology and Immunology, Weill Cornell Medical College, New York, NY 10065, USA

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ABSTRACT

Malaria parasites replicate asexually within their mammalian hosts as haploid cells and are subject to DNA damage from the immune response and chemotherapeutic agents that can significantly disrupt genomic integrity. Examination of the annotated genome of the parasite *Plasmodium falciparum* identified genes encoding core proteins required for the homologous recombination (HR) pathway for repairing DNA double-strand breaks (DSBs), but surprisingly none of the components of the canonical non-homologous end joining (C-NHEJ) pathway were identified. To better understand how malaria parasites repair DSBs and maintain genome integrity, we modified the yeast I-SceI endonuclease system to generate inducible, site-specific DSBs within the parasite's genome. Analysis of repaired genomic DNA showed that parasites possess both a typical HR pathway resulting in gene conversion events as well as an end joining (EJ) pathway for repair of DSBs when no homologous sequence is available. The products of EJ were limited in number and identical products were observed in multiple independent experiments. The repair junctions frequently contained short insertions also found in the surrounding sequences, suggesting the possibility of a templated repair process. We propose that an alternative end-joining pathway rather than C-NHEJ, serves as a primary method for repairing DSBs in malaria parasites.

INTRODUCTION

Malaria remains the number one killer of children aged less than 5 years in sub-Saharan Africa. Though a reduction in malarial deaths in select African countries has recently been reported and plans have been made for malaria eradication, it is widely recognized that the tools to achieve this goal have yet to be developed. Malaria is caused by eukaryotic parasites of the genus Plasmodium, with *Plasmodium falciparum* causing the most severe form of human disease. With the emergence of artemisinin resistance (1) and the continued limited efficacy of vaccines, further study of how these parasites adapt to stresses from their environment, both immune and drug pressures, are warranted.

Malaria parasites, like all pathogens, must rely on DNA repair pathways to resist genomic damage inflicted by multiple sources, including various metabolites, errors during replication and the immune response of the host. The release of substances by immune cells that cause oxidative damage, including reactive oxygen and nitrogen species, are known to cause DNA damage (2). Infectious organisms may also potentially benefit from 'mistakes' in repair as such errors often generate diversity that can contribute to the development of drug resistance or other means of accelerated adaptation to the host (3). There are two well-characterized mechanisms for repair of DNA double-strand breaks (DSBs), canonical non-homologous end joining (C-NHEJ) and homologous recombination (HR) (4). In general, higher eukaryotes and prokaryotes have profoundly different responses to DSBs; while higher eukaryotes have long been known to use C-NHEJ as the predominant method to repair a DSB, prokaryotes use HR almost to exclusion (4). HR-based repair is considered to be largely error free as a template is used to guide repair while C-NHEJ is more likely to introduce mutations.

Lower eukaryotes, including protozoans, have been shown to possess a mixed 'toolkit' of repair processes (5–7). C-NHEJ has been described in *Toxoplasma* (8–10), while in trypanosomes, HR is the predominant pathway used for DSB repair (11,12). In addition, some DNA repair proteins have been shown to have alternative

*To whom correspondence should be addressed. Tel: +1 212 746 4976; Fax: +1 212 746 8587; Email: kwd2001@med.cornell.edu
Present address:
Elizabeth A. Lawrence, Merck and Co. Inc., Durham NC, 27712, USA.

functions as their primary role. For example Ku 70/80, enzymes involved in end joining (EJ), are encoded in the trypanosome genome, however their primary role appears to be in telomere maintenance and not DNA DSB repair (13,14). Recently alternative pathways to Ku-mediated C-NHEJ have been described (15–17) and are collectively referred to as A-NHEJ. Various studies have identified an alternative NHEJ pathway based on 'microhomology' (12,15). A-NHEJ has a completely different DNA repair toolkit and functions independently of the components of C-NHEJ. Both types of NHEJ restore genomic integrity but with different mutation propensities, thus the sequence of the repaired DNA and the resulting mutations shed light on the type of pathway used to repair the DSB.

Plasmodium is remarkable in that even though it is haploid for much of its lifecycle, including the pathogenic erythrocytic stage, and thus does not possess the homologous sequences required for HR, the proteins required for C-NHEJ, including Ku 70/80 and DNA ligase 4 have not been identified either bioinformatically or biochemically (18,19). The apparent loss of the C-NHEJ pathway from malaria parasites appears to be a relatively recent evolutionary event given that the closely related parasite *Toxoplasma gondii* displays a robust C-NHEJ response to DSBs and the required proteins are easily identifiable within the *T. gondii* genome (8). Further evidence suggesting that C-NHEJ is not active in malaria parasites includes the inability of parasites to close a linearized plasmid after transfection and the integration of exogenous DNA exclusively at sites of sequence homology (20). After countless genetic manipulation of parasites in numerous independent laboratories, no examples of DNA repair events indicative of C-NHEJ have been reported. Thus it is unclear how the parasite responds to DSBs and maintains its genome integrity during asexual development in its mammalian host.

In this study, we adapted a yeast endonuclease expression system for use in cultured *P. falciparum* to study alternative pathways for repair of DSBs during asexual replication. As predicted from genomic annotations, parasites appear to possess a typical HR-mediated repair pathway and readily undergo gene conversions to repair breaks within sequences that share significant identity elsewhere in the genome. When DSBs were created within unique genomic regions, parasites were able to repair the lesions using a form of EJ, despite the apparent lack of proteins required by the C-NHEJ pathway. Analysis of the repair junctions identified a mechanism of repair that included resection of single-strand overhangs and insertion of short stretches of synthesized nucleotides at the site of the break. These characteristics are similar to that previously described as synthesis-dependent microhomology-mediated end joining (SD-MMEJ) (21), implicating a derivation of this pathway as a method for repairing DSBs by malaria parasites.

MATERIALS AND METHODS

Plasmodium falciparum culture and transfection

Plasmodium falciparum lines were cultured at 5% hematocrit in RPMI 1640 medium, 0.5% Albumax II (Invitrogen), 0.25% sodium bicarbonate and 0.1 mg/ml gentamicin. Parasites were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide and 90% nitrogen. The parasite line 3D7att was obtained from MR4 (MRA-156, MR4, ATCC Manassas Virginia, VA, USA). Parasites were transfected using 'DNA loaded' red blood cells as previously described (22). For stable transfections, parasites were cultured in media containing either 40 ng/ml WR99210 (Jacobus Pharmaceuticals, Plainsboro, NJ, USA), 400 μg/ml neomycin (G418, Sigma, St. Louis, MO, USA), 1.5 μM DSM (Vaidya laboratory, Drexel University) or 2 μg/ml of blasticidin (Invitrogen- Life Technologies, Grand Island, NY, USA).

Plasmid creation

I-SceI cut sites

The original pLN-ENR-GFP (23) was obtained from MR4 (MRA-156, MR4, ATCC Manassas Virginia) and modified to create the different parasite lines. The *enoyl-acyl carrier reductase* gene (PF3D7_0615100) was replaced with various modified cassettes using the AvrII and BsiWI (New England Biolabs, Ipswich, MA, USA) cut sites. The *enoyl-acyl carrier reductase* gene containing the I-SceI recognition site was created by amplifying the coding region from the original pLN-ENR-GFP plasmid, which contains the gene from the Dd2 parasite line. The first segment of the gene was amplified using a primer to the 5′ end and a reverse primer that included the 18-bp I-SceI recognition sequence followed by a Not I site. The second fragment was amplified using primers that included Not I and BsiW I sites. The complete gene, including the I-SceI site, was assembled by ligating the fragments into the pLN-ENR-GFP vector using the Avr II, Not I and BsiW I compatible ends.

The plasmid containing the *var* gene fragment was created by amplifying a fragment of PF3D7_0223500, a B type *var* gene found on Chromosome 2. The I-SceI recognition site was inserted using site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kits, Agilent Technologies, Santa Clara, CA, USA). Cassettes with a *enoyl-acyl carrier reductase* gene containing both the I-SceI recognition site and introduced SNPs (2% sequence difference and 20% sequence difference) were obtained from Geneart (Life Technologies, Grand Island, NY, USA). The *Guassia* luciferase gene containing the I-SceI recognition site was created by Genewhiz (South Plainfield, NJ, USA). All cassettes were then inserted into the pLN-ENR-GFP plasmid at the Avr II and BsiW I sites.

I-SceI expression plasmid

The expression plasmid pHBIRH (24) was modified to create an I-SceI expression plasmid. The gene encoding the I-SceI endonuclease was amplified from the pCBASce plasmid (25). The protein encoded in this construct is fused to a 5′-nuclear localization signal. This sequence was cloned into the expression vector using the Not I and Sac I sites. The *blasticidin S deaminase* cassette was replaced by the neomycin resistance cassette. To add

the destabilization domain we utilized the DD and 3′-HSP 86 3′-UTR from pEcDamLo (26).

An alternative expression plasmid was made replacing the neomycin resistance cassette with the DHOD gene conferring resistance to atovaquone. The DHOD was amplified from pUF1 (27) and cloned into the BamH I and Hind III sites. The bi-directional promoter was replaced by the PcDT 5′- and hsp86 5′-promoters amplified from pINT (23). pINT was obtain from MR4 (MRA-156, MR4, ATCC Manassas Virginia).

Creation of transgenic parasites and I-SceI induction

Parasite lines harboring the I-SceI endonuclease recognition site were created using the mycobacteriophage Bxb1integrase system in order to obtain a single copy integration of the plasmid using the published protocol (23). Once stable integrations were selected, parasites were grown off neomycin and allowed to shed the integrase plasmid. This line was then subcloned to select for parasites that no longer carried pINT. This enabled the use of neomycin to select for the I-SceI expressing plasmid. The *Gaussia* (I-SceI) and ENR (2%) lines were not subcloned and therefore the alternative I-SceI expressing plasmid with DHOD as the selectable marker was used. Single copy integration of all constructs was validated by PCR using primers previously published (23). Southern blotting (Figure 1) for the initial ENR(SCE) lines was performed using standard protocols (28). Once parasite lines were established that had both the integrated cut site and the I-SceI expression plasmid, gDNA was extracted and plasmid rescue was performed to verify the I-SceI cassette had not been rearranged. If intact, the parasites were then grown in the presence of 0.5–1 μM Shield1 (Clontech, Mountain View, CA, USA). Induction of the endonuclease under Shield1 was verified by western blotting of extracts obtained from parasites grown in the presence or absence of Shield1. Late-stage synchronized parasites were harvested and the red cells were lysed in PBS containing 0.05% saponin (Sigma). After centrifugation, the parasite pellet was resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1) containing a protease inhibitor cocktail. The samples were solubilized by sonication in SDS sample buffer prior to electrophoretic separation using Mini-Protean gels (Bio-Rad). Proteins were transferred to nylon membrane using a BioRad electroblotting apparatus and the manufacturer's protocols. The membrane was probed with the anti-DD domain antibody (Clontech) at a dilution of 1:500 as recommended. Membranes were stripped and reprobed with anti-PF39 anitbodies to verify equal loading (29).

Analysis of repair products

Genomic DNA was extracted from parasite cultures after 20 days of growth in the presence of Shield1. Repair products were amplified by PCR using forward primers to either the 5′-end of the inserted gene containing the I-SceI recognition site or to the sequence flanking the insert. Reverse primers were based in the GFP cassette. PCR products were then subcloned in Topo-Blunt (Invitrogen) and sequenced.

Generation of parasite subcultures

To obtain independently derived repair products, replicate subcultures were obtained using a 96-well plate format. Precise parasite concentrations of cultures of the various transgenic lines were determined using a hemocytometer. Dilution series in 96-well plates were then used to obtain 200 μl cultures containing 10 parasites, 100 parasites or 1000 parasites per well. After plating, parasites were grown in the presence of Shield1 to initiate DNA cleavage and repair. Culture plates were kept in a modular incubator chamber at 37°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Ten wells at each concentration were plated. Media was changed and cultures checked by smear every 48 h until growth was detected in all wells. To avoid overgrowth, the parasitemia in each well was maintained between 0.5% and 3%. The culture media in each well was replaced with fresh media containing Shield1 every other day. When the parasitemia in all wells had reached 1–5%, gDNA was obtained for PCR analysis using a rapid boiling method. Briefly, the media was removed from each well and the red cells lysed by the addition of 150 μl of water. The samples were then boiled at 95°C for 10 min. PCR was then performed across the I-SceI cut site to analyze repair products.

RESULTS

Development of a system to generate site-specific DSBs in cultured malaria parasites

To study DNA DSB repair in *P. falciparum*, we adapted a site-specific endonuclease system previously used to study DSB repair in several model organisms. To create our initial set of parasite lines, we used the mycobacteriophage Bxb1 integrase system (23) to obtain site-specific integration of a single copy of a plasmid containing a fragment of the *enoyl-acyl carrier reductase* (ENR) gene (PF3D7_0615100) with a 18-bp I-SceI endonuclease recognition site inserted within the coding region. This yielded a parasite with two homologous copies of a gene with ~0.6% variation due to single nucleotide polymorphisms (SNPs) (Figure 1A). Once created, these parasites were then cotransformed with a plasmid expressing the I-SceI endonuclease (30). The I-SceI endonuclease is a yeast endonuclease that is highly specific for an 18 bp recognition site (Figure 1B) and has been used extensively in mammalian, yeast and bacterial systems to study DSB repair (31,32). Because the I-SceI recognition sequence is not naturally found in the *P. falciparum* genome, expression of this enzyme caused a single, unique DSB within the engineered *enoyl-acyl carrier reductase* gene. Since the endonuclease recognition site was integrated into the genome, the break must be repaired for cell viability.

All initial attempts to create transgenic parasites carrying both the I-SceI recognition sequence and expressing the endonuclease yielded no viable parasites, presumably due to the combined inefficiency of parasite transfection and DSB repair. In contrast, all attempts at

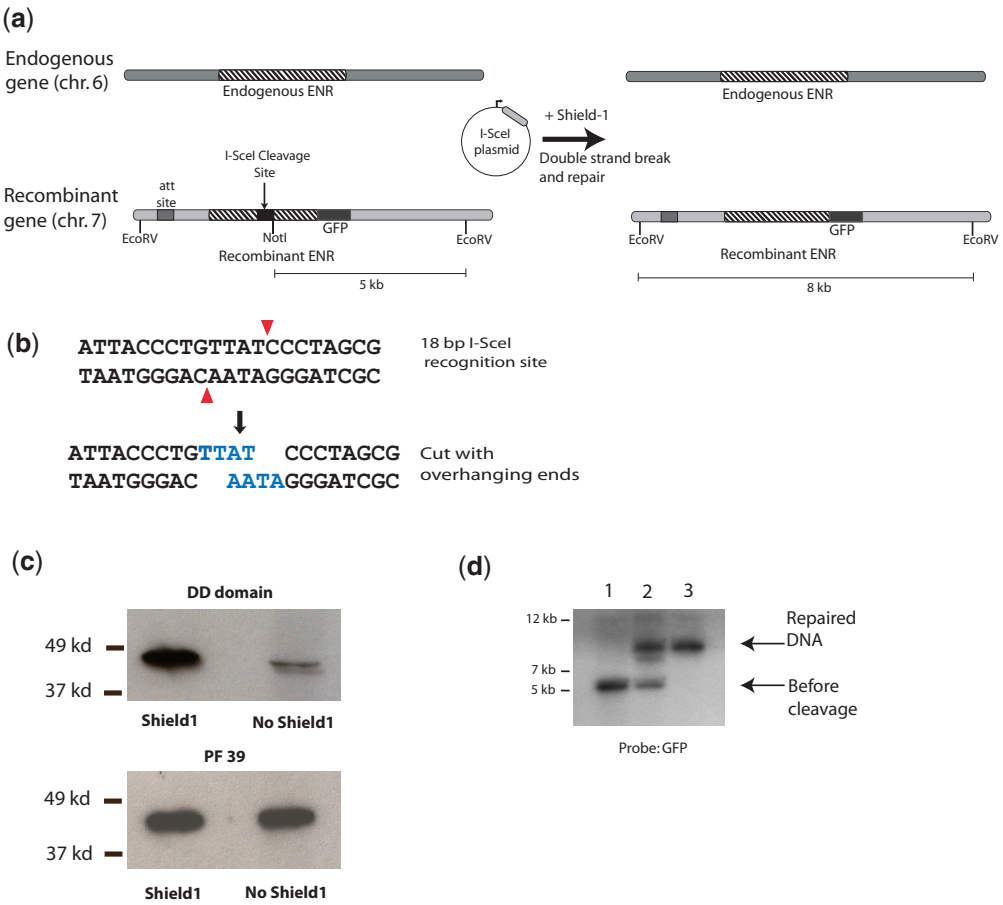

Figure 1. A system for inducing targeted, DSBs in *P. falciparum*. (**a**) On the left is shown the endogenous *enoyl-acyl carrier reductase* gene (ENR) on chromosome 6 (top) as well as the duplicated sequence containing the I-SceI site and inserted into the att site on chromosome 7 (bottom). Note that digestion with Not I/EcoR V results in a 5-kb fragment containing the GFP tag. On the right is shown the same sequences after induction of the I-SceI endonuclease. Cleavage and repair leads to gene conversion and loss of the Not I site, resulting in an 8-kb fragment containing the GFP tag after digestion with Not I/EcoR V. (**b**) The I-SceI recognition and cleavage site is shown. Note that cleavage results in a 4 bp 3′-overhang (blue or gray text). (**c**) Western blot showing stabilization of the I-SceI endonuclease upon treatment with Shield1. Extracts from parasites grown in the presence or absence of Shield1 were separated by SDS–PAGE, blotted onto nylon membrane and probed with antibodies specific for the destabilization domain that is fused to the endonuclease (top panel). To control for loading, the blot was stripped and reprobed with antibodies to the protein Pf39 (bottom panel). (**d**) Southern blot hybridized with a probe to GFP. Lanes 1, 2 and 3 show genomic DNA extracted from parasites after 0, 10 and 20 days of growth in the presence of Shield1, respectively.

creation of parasite lines that harbored our expression plasmid but not the cleavage site were successful. To avoid this problem, we employed an inducible expression system that relies on fusion of a destabilization domain to the protein of interest (33). Using this system, we fused the destabilization domain to the I-SceI endonuclease, thus downregulating the endonuclease activity sufficiently to create stably transformed lines. As seen in Figure 1C, upon addition of Shield1 the endonuclease was stabilized, resulting in a substantial increase in the steady state expression level of I-SceI. The ability to upregulate endonuclease expression and induce the generation of a single, site-specific DSB allowed us to systematically analyze the products of repair.

Repair of DSBs via HR and the generation of gene conversions

Adjacent to the I-SceI recognition site within the *enoyl-acyl carrier reductase* sequence is a Not I cleavage site. If a DSB was in fact created by the endonuclease and subsequently repaired by HR, generating a gene conversion, the Not I site was expected to be lost from the final repair product (Figure 1A). Southern blots of DNA extracted from parasites at different time points after addition of Shield1 show the predicted shift in the size of the DNA fragment hybridizing to a probe within the targeted gene, consistent with repair by gene conversion (Figure 1D). Establishment of a parasite population in which there was complete loss of the original insert containing the I-SceI site took approximately 20 days, suggesting that either induction of endonuclease activity by addition of Shield1 is not efficient, or that repair of the break frequently does not disrupt the I-SceI site and allows for repeated cutting/repair cycles until the I-SceI site is eventually lost. Importantly, repair of DSBs by EJ frequently results in addition/loss of individual bases at the site of repair. Thus while repair by HR results in loss of the Not I site, repair by EJ would prevent

additional cleavage events and stabilize the insert, but would leave the Not I site intact. However, by 20 days after addition of Shield1 to the culture, the Not I site was no longer detectable by Southern blot, suggesting that HR was the dominant, if not exclusive, repair pathway utilized to repair a break in this context.

To confirm that the DSBs generated within the *enoyl-acyl carrier reductase* sequence were in fact repaired through HR-mediated gene conversion events, after 20 days of Shield1 treatment, the repair junction was amplified and sequenced. The sequence data from the repaired gene showed a reinstated *enoyl-acyl carrier reductase* gene and complete elimination of the inserted 18 bp I-SceI recognition sequence (Figure 2A and B). The presumed 'donor' sequence for HR in this instance was the endogenous *enoyl-acyl carrier reductase* gene located on chromosome 6, which includes several SNPs when compared with the sequence surrounding the I-SceI site. Analysis of PCR amplified repair junctions from two independently transfected lines, each containing $\sim 1.5 \times 10^6$ parasites at time of endonuclease induction, as well as Southern analysis of parasite cultures that had undergone break and repair, exclusively detected a hybrid *enoyl-acyl carrier reductase* gene with an intact open reading frame at the site of repair. Consistent with HR as the method of repair, the donor sequence was found to be unchanged. The two copies of *enoyl-acyl carrier reductase* were located on different chromosomes and from different parasite isolates (accounting for the SNPs) yet repair by gene conversion was the only mechanism detected. No sequences with base-pair deletions or modifications consistent with NHEJ were obtained in these transgenic parasites after cleavage and repair of the target sequence.

Multicopy gene families also use gene conversion for repair of DSB

We next established a parasite line with the I-SceI recognition site contained within a *var* gene fragment inserted at the Bxb1 integration site. The *var* gene family encodes PfEMP1, a protein made by the parasite and exported to the membrane of the infected red blood cell. This protein is key to the pathogenesis of *P. falciparum* malaria and has been implicated as the main determinant in antigenic variation. There are approximately 60 *var* genes located throughout the parasite's genome, with a single gene expressed while the remainder are maintained in a silent state. The genes are highly variable, but contain blocks of sequence similarity that could act as templates for semi-homologous repair. In depth sequence comparisons of the gene family identified numerous probable examples of previous gene conversion events, suggesting repair of DSBs by HR could provide a way for the parasite to diversify this important gene family (34). We therefore hypothesized that induced breakage of a *var* gene fragment by our endonuclease system might result

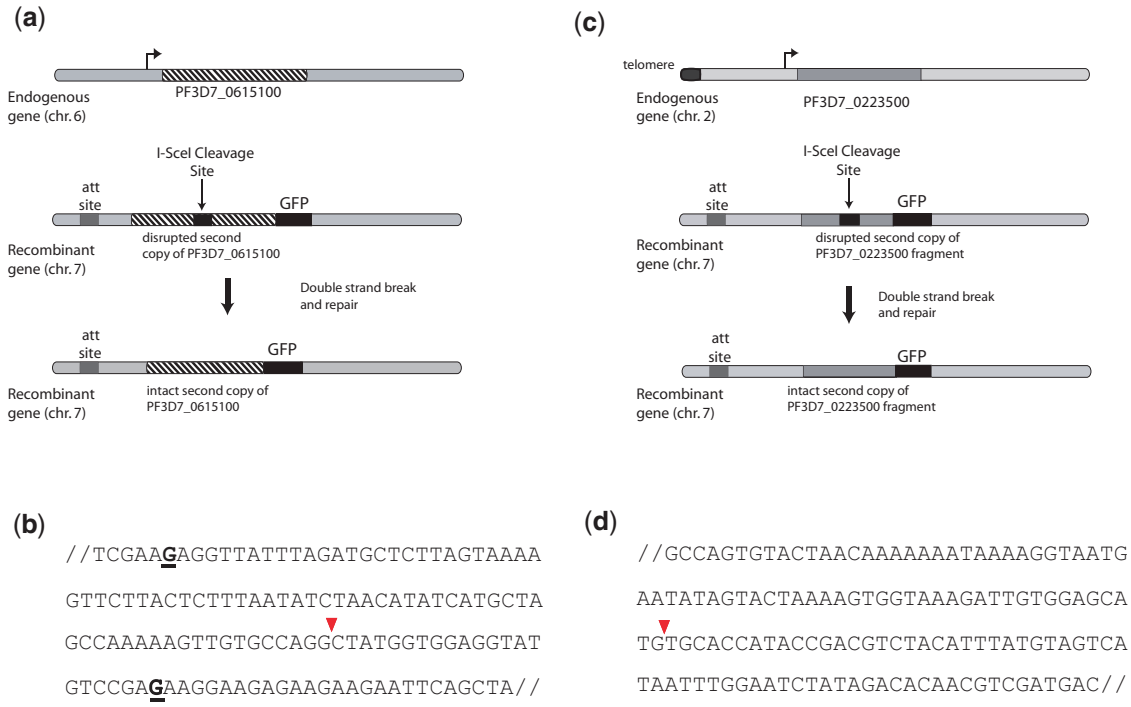

Figure 2. HR-mediated repair leading to gene conversion events. A fragment from either the *enoyl-acyl carrier reductase* gene (PF3D7_0615100, ENR) on chromosome 6 (**a**) or from a *var* gene (PF3D7_0223500) within the subtelomeric region of chromosome 2 (**c**) was inserted at the att site on chromosome 7. Both duplicated fragments contained an I-SceI site inserted near the middle of the sequence. Break and repair of the duplicated fragments elicits repair by gene conversion resulting in removal of the I-SceI sites. (**b**) The sequence of the duplicated PF3D7_0615100 sequence after cleavage and repair. The arrowhead shows the location of the 18-bp I-SceI sequence prior to the gene conversion event. Nucleotides in bold and underlined indicate SNPs between the endogenous gene and the fragment inserted at the att site. (**d**) The sequence of the duplicated PF3D7_0223500 sequence after cleavage and repair. The arrowhead shows the location of the 18-bp I-SceI sequence prior to the gene conversion event.

in multiple repair products reflecting the use of alternative *var* genes as templates for repair.

For this study we amplified a fragment of PF3D7_0223500, a *var* gene located in the subtelomeric region of Chromosome 2. The I-SceI recognition site was inserted within a relatively conserved part of the 5′-end of exon 1, and this hybrid-gene product was inserted into the Bxb1 site on chromosome 7 (Figure 2C). Addition of Shield1 to a bulk culture of $\sim 1.5 \times 10^6$ parasites for three weeks resulted in cleavage and repair as expected, and the repair products were amplified and sequenced. While the genome contains ~60 intact *var* genes that could theoretically have been used as templates for repair, just as in our experiments with *enoyl-acyl carrier reductase,* we only obtained a single repair product. The endonuclease site was completely resected and only a reinstated PF3D7_0223500 gene fragment was found (Figure 2D). To determine if alternate, less frequent repair products could be obtained by inducing break and repair in multiple, smaller populations of parasites, we independently induced endonuclease activity in 10 subcultures of the transgenic line (each diluted to 10 parasites per subculture at the time of induction). However the subcultures also all yielded the same repair product. We were unsuccessful in inducing endonuclease activity in subcultures initiated with single parasites do to the effect of Shield1 on red cell stability over the additional time required to obtain sufficient parasite growth for DNA extraction. In all experiments, there was no evidence of recombination with the *var* gene cluster 100 kb away on the same chromosome or other closely related *var* B family members found in the subtelomeric domains of other chromosomes. Thus, at least in the context of our inducible cleavage system and the *var* gene sequence that we chose to use for the assay, the sequence requirement for the selection of templates for repair by HR appears to be sufficiently strict to make isolation of repair products that used non-identical templates below our ability to detect.

Evidence for an EJ pathway in *P. falciparum*

As mentioned above, to date none of the components of the C-NHEJ pathway have been identified in malaria parasites, thus how parasites maintain genome integrity and repair DSBs while haploid remains unresolved. In an attempt to detect the existence of an EJ pathway in *P. falciparum*, we modified our transgenic parasite lines so that an I-SceI recognition site was inserted into a non-*falciparum* gene (the gene encoding *Gaussia* luciferase) for which a donor sequence for HR was not provided. This hybrid gene product was again inserted into the Bxb1 site on chromosome 7, and the transgenic parasites were then transfected with the I-SceI expression plasmid (Figure 3A). Since no homologous template sequence was provided, some form of EJ was the only alternative for restoring genome integrity and cell survival. After induction of the endonuclease, parasites that had grown in the presence of Shield1 for 3 weeks were obtained, genomic DNA was extracted and the repair products were amplified and sequenced. Surprisingly only two distinct repair products were identified (Figure 3B). This indicates that parasites do in

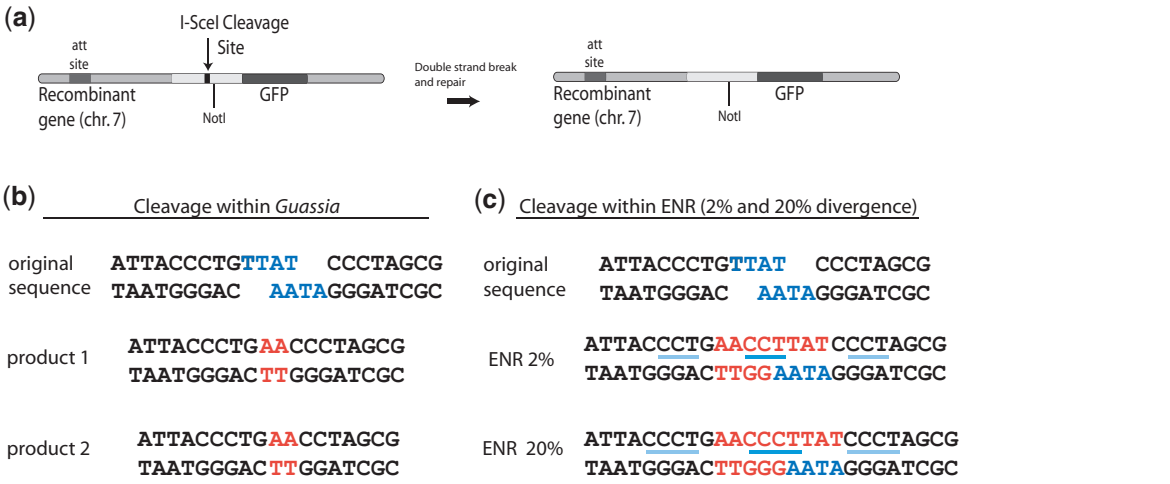

Figure 3. EJ-mediated repair of a DSB. (**a**) A fragment from either the *Gaussia* luciferase gene or the *enoyl-acyl carrier reductase* (ENR) gene containing either 2% or 20% sequence divergence from the endogenous copy was inserted into the att site on chromosome 7. All of the inserted fragments contained the I-SceI recognition site near the middle of the insertion. Induction of the I-SceI endonuclease with Shield1 led to a DSB and repair, resulting in disruption of the recognition site. Note that repair by EJ results in the Not I site being maintained. (**b**) The sequences of the repair products for *Guassia* luciferase are shown. Both strands of each DNA molecule are represented. The original I-SceI site is displayed on top, with the break shown as a gap in the sequence and the single-stranded overhangs shown in blue/gray type. Two products of repair are shown. Note that the single stranded stretches have been deleted in the repair products and an AA doublet inserted (highlighted). Also note that a single C was deleted in product 2. (**c**) The sequences of the repair products for ENR fragments are shown. The original I-SceI site is displayed on top, with both strands of the DNA molecule shown and the single-strand overhangs shown in blue/gray. Two products of repair are shown, one observed when the ENR sequence diverged from the endogenous copy by 2% and the second when the sequence diverged by 20%. Base pairs that have been synthesized as part of the repair process are highlighted. Note that the TTAT on the top strand was likely newly synthesized during the repair process since it resides downstream of the inserted AACC stretch. Potential template sequences for repair are underlined with light blue/gray and the corresponding duplicated sequence in dark blue/gray.

fact have the ability to repair DSBs through an EJ pathway and that they can repair DSBs without major alterations to the sequences surrounding the break. The sequences of the repair products indicate that the 3′-overhangs left by the endonuclease upon cleavage were resected followed by insertion of 2 nt prior to ligation. It is interesting to note the double A insertion found in both repair products. The assay would not detect any very large deletions that extended beyond our PCR primers and thus deleted the entire recombinant cassette, however given that we never detected any deletions outside the actual cleavage site, this seems unlikely unless the parasite only utilizes deletions >1 kb for repair.

Given the small number of repair products and their close sequence similarity, we hypothesized that the same products of repair were generated numerous time independently. To explore this possibility, we verified by PCR and sequencing that the cleavage site was intact prior to addition of Shield1 in our initially transfected line. We then generated thirty subcultures of parasites by limiting dilution in 200 µl volumes. Subcultures were initiated with either 10, 100 or 1000 parasites, then exposed to Shield1 until parasitemia levels reached ∼5% in all wells, at which time DNA was extracted and the products of repair were amplified and sequenced. Despite the fact that only the intact cleavage site was detectable prior to dilution of the parasites and exposure to I-SceI, only a single product of repair was obtained from all of the independent subcultures, indicating that the same repair product was likely generated repeatedly. The recurrent isolation of two almost identical products of repair suggests that the repair process is templated. Though the resulting total number of repair products characterized in these experiments is small, they confirmed that the parasites had indeed utilized a form of EJ to repair the DSB, thereby demonstrating for the first time the existence of this type of DNA repair in malaria parasites.

Choice of HR versus EJ to repair DSBs

HR is known to be the more exact and error free method of DSB repair, yet it requires the presence of a homologous sequence elsewhere in the genome to act as donor. There is variability in different species in the degree of sequence divergence tolerated between the 'donor' sequence and the site of the break (35,36), however in general, HR is suppressed with >1% sequence divergence (37). In the mammalian system, specific aspects of gene conversion have been explored using the I-SceI recognition sequence inserted into different modified genes (37). By manipulating the SNPs between a cleaved and a donor sequence, it has been possible to define the characteristics that determine which pathway is used for repair.

To determine the degree of sequence identity required by *P. falciparum* to utilize HR rather than EJ to repair a DSB, we utilized the *enoyl-acyl carrier reductase* gene described above. As shown in Figure 1, in our initial experiments, the endogenous 'donor' sequence differed from the sequence surrounding the I-SceI site at several SNPs, resulting in an overall difference of ∼0.6% across the length of the insert. To determine at what degree of sequence divergence parasites will switch to EJ for repair rather than HR, we created synthetic constructs in which the sequence surrounding the I-SceI site included either 2% or 20% sequence divergence. The resulting products of repair were then amplified and sequenced to determine the pathway used by the parasites to repair the break. As described above, when the sequences diverged by only 0.6%, parasites that had repaired the DSB were easily obtained and HR was the exclusive pathway detected. However, when the sequences diverged by either 2% or 20%, repair was similar to that observed when the I-SceI site was placed within the *Gaussia* sequence. PCR amplifications of DNA obtained from three independent transfections (including the use of two different expression plasmids using different selectable markers) using three alternative primer pairs only recovered two distinct repair products (Figure 3C). These products displayed the same double A insertion at the site of the break as previously observed, but also insertions of an additional 4 or 5 bp. This analysis indicates that similar to mammals and yeast, when the sequence surrounding the site of a DSB diverges >∼1% from sequences found elsewhere in the genome, *P. falciparum* primarily uses EJ to repair DSBs. This is despite the proposed lack of a C-NHEJ pathway that dominates EJ in higher eukaryotes. It is interesting to note the remarkably few number of repair products that were obtained per construct, even from completely independent transfection experiments. It is also noteworthy that repair within the divergent ENR constructs appears not to have resected the 4-bp overhang on one strand of the cleaved DNA (Figure 3C). This resulted in the apparent synthesis of a TTAT sequence after insertion of the AACC sequence at the site of the break. This was not observed within the repair products found when the break was induced within the *Guassia* sequence.

Comparison of HR and EJ

In the pseudo-diploid lines that utilized HR for repair, we were easily able to obtain transfected parasites containing both the cleavage site within their genome and carrying the I-SceI expression plasmid. In contrast, when parasites were not provided the opportunity to use HR for repair, our ability to obtain transfected lines was greatly reduced. Of the combined 24 independent transfection attempts in which EJ was the only means for repair, 19 were unsuccessful in generating parasite lines that contained both the endonuclease expression plasmid and the cleavage site within their genome. In approximately half of these attempts, no viable parasites were obtained after selection for transfection with the I-SceI expression plasmid. Simultaneous transfections of parasites that did not harbor the recognition site within their genomes were successful, indicating that the expression plasmid was functional and the transfection conditions were suitable. In the remainder of the failed transfections, parasites were obtained that carried an I-SceI expression plasmid in which either the cassette encoding the endonuclease or the associated promoter had been deleted, thus preventing

expression of the enzyme. Our inability to efficiently generate transfected lines that express the endonuclease when its recognition site is within a unique part of the genome suggests that DSB repair is much less frequent when the parasites cannot use HR and are instead forced to rely on some form of EJ for repair.

DISCUSSION

Evidence for gene conversion events have been previously reported in *P. falciparum* via analysis of the progeny of a genetic cross and by large-scale comparisons of sequences from variant multicopy gene families (34,38,39). Here we provide the first description of inter-chromosomal GC events that were experimentally induced and therefore definitively shown to occur in the haploid erythrocytic stage of the parasite's life cycle. Importantly, we only observed GC events when we supplied a template for repair that contained significant sequence identity with the site of the break (<2% sequence divergence). Furthermore, our experiments with a *var* fragment showed that there appears to be a preference for a nearly exact sequence match even when alternative, more divergent *var* gene templates are located in the same region of the chromosome as the break. The repair events generated by GC appeared to occur with high fidelity and no changes from the donor sequence were detected.

In most higher eukaryotes, of the two EJ pathways, C-NHEJ is dominant, with A-NHEJ pathways serving as less efficient, less accurate backup pathways (15). A-NHEJ is typically only readily observed in model organisms when the C-NHEJ pathway has been disrupted in some way (40). As previously mentioned, malaria parasites are unique in that orthologs of several proteins required for C-NHEJ cannot be identified within the parasite's genome using standard computational approaches, despite their presence within the genomes of closely related apicomplexans like *Toxoplasma gondii*. Thus they appear to be naturally deficient in C-NHEJ and potentially must rely on alternative pathways to repair DSBs (18). This is consistent with the fact that we only recovered products of HR when a DSB was induced in parasites in which a homologous sequence for repair was present. Various proteins have been proposed to be involved in the different repair pathways collectively referred to as A-NHEJ, some of which have been identified in the *P. falciparum* genome. However, most of these proteins also serve other nuclear functions, therefore it was not previously known whether malaria parasites possessed a functional A-NHEJ pathway. Considering the identifiable repair proteins encoded in the *P. falciparum* genome, we hypothesized that parasites would repair DSBs with resection and ligation at sites of microhomology, however the observed repair products were more consistent with repair by a templated EJ process. Despite multiple independent transfections, repeated induction of endonuclease activity in separate subcloned cultures and PCR amplifications using alternative primer pairs, a very limited cohort of repair products were obtained from any individual construct. Together these findings are most consistent with a templated mechanism of repair that is likely to generate the same or very similar products repeatedly. The difficulty in obtaining successful transfections that required EJ also suggests that this repair mechanism is much less efficient than HR, although more complete studies will be required to fully understand the relative efficiencies of DNA repair pathways in this organism.

The various pathways collectively known as A-NHEJ differ from C-NHEJ in several respects including end processing, kinetics of repair and type of associated mutations in the repaired product (38). C-NHEJ is associated with small insertions and deletions whereas A-NHEJ is more often associated with EJ at areas of microhomology, often leading to large deletions and some chromosomal rearrangements. More recently, a single underlying mechanism has been proposed to account several different types of repair products, including those that display blunt joins, junctional microhomologies and short insertions/deletions (21). This mechanism employs a non-processive DNA polymerase to create microhomology at the site of a DSB, which then allows annealing, ligation and repair. It is referred to as SD-MMEJ (21). Hallmarks of this type of repair are single base-pair deletions or insertions at or near the site of the break, as well as short stretches of sequence insertions that appear to have been templated from regions very close to the break. Analysis of the repair junctions found at the sites of I-SceI cleavage in all of our constructs that had undergone EJ allowed us to investigate if SD-MMEJ might also account for EJ in malaria parasites. In all cases, a similar set of additional base pair had been inserted at the site of the break, suggesting that the repair process was templated and therefore involved a polymerase capable of synthesizing short stretches of DNA (Figure 3).

While the molecular components of the SD-MMEJ pathway are largely unknown, this mechanism requires a polymerase activity to synthesize short stretches of DNA at the site of the break. This newly synthesized DNA serves as the source of the microhomology that facilitates annealing and ligation. In Drosophila, there is evidence that DNA polymerase theta serves this function (21,41). Examination of the *P. falciparum* genome has identified one polymerase that contains the Pol A superfamily domain (PF3D7_0625300) found in pol theta, but the full function of this polymerase has yet to be explored. The remaining identified DNA polymerases appear to be limited to the B family polymerases (alpha, delta, epsilon and zeta). We and others failed to identify any family X polymerases, which is consistent with the lack of other components of C-NHEJ, as well as the published lack of short patch repair in base excision repair (BER)(42).

There is evidence that different *P. falciparum* isolates have varying abilities to repair DNA lesions (43). However these reports have been limited to studies of mismatch repair and the generation of point mutations (44,45), and have not investigated possible variation in the ability of parasites to repair DSBs. Given the increasing availability of whole-genome sequence data from numerous parasite isolates, investigations into DSB repair in different lines could be used to identify genes involved in this pathway. It is also likely that the

extreme AT content of *P. falciparum* has influenced how the parasites repair DSBs as well as other genomic lesions. It is worth noting that the I-SceI recognition sequence includes two GC-triplets on opposite sides of the resulting DSB. Whether this relatively high GC content influenced the type of repair products we recovered is not known. An alternative method for introducing site-specific DSBs within a more AT-rich stretch will be required to investigate this possibility.

As mentioned briefly, the hypervariable, multicopy gene families involved in immune invasion display blocks of sequence homology that are indicative of frequent gene conversion events. For example, considerable sequence mosaicism has been found within members of the *var* gene family that encode the primary surface antigen displayed on the surface of red blood cells infected by *P. falciparum* (34,38,46). This mosaicism is thought to be a product of 'shuffling' of functional domains through both recombination during meiosis as well as gene conversion during asexual replication (34,38,47). These gene conversions presumably are the result of HR repair of DSBs within individual members of the family. The shuffling of sequences between *var* genes does not however appear to be random, but rather large-scale sequence analysis of both laboratory strains and field samples has revealed a highly structured network containing putative groups of genes that appear to preferentially recombine (48–50). The groups have been termed A, B and C types and are defined by chromosomal position, domain structure, direction of transcription and the surrounding non-coding regions (49,50). *var* genes thus appear to be diversifying separately among these three different groups through gene conversion events generated via HR. Interestingly, while members of a particular *var* group display significant sequence similarity, it is generally well below the 98% sequence identity shown in this study to be required for HR to be utilized rather than EJ. Therefore additional constraints must influence the choice of HR versus EJ when a DSB is encountered within a *var* gene. There is growing evidence that higher order chromosomal architecture exerts just as profound an influence on DNA repair that it does on nuclear processes like transcription and DNA replication (51,52). Both of these characteristics have been shown to influence the choice of repair pathways used to repair DSBs. Thus in addition to contributing to regulation of gene expression, the unique chromatin structure and genome organization of these large multi-copy gene families might also contribute to their on-going diversification. Extension of the studies described here describing the choice between HR and EJ in response to DSBs at *var* loci will shed light on this important aspect of genome biology in malaria parasites.

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