High-efficiency transformation of *Plasmodium falciparum* by the lepidopteran transposable element piggyBac

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Functional analysis of the *Plasmodium falciparum* genome is restricted because of the limited ability to genetically manipulate this important human pathogen. We have developed an efficient transposon-mediated insertional mutagenesis method much needed for high-throughput functional genomics of malaria parasites. A drug-selectable marker, human dihydrofolate reductase, added to the lepidopteran transposon piggyBac, transformed parasites by integration into the *P. falciparum* genome in the presence of a transposase-expressing helper plasmid. Multiple integrations occurred at the expected TTAATA target sites throughout the genome of the parasite. We were able to transform *P. falciparum* with this piggyBac element at high frequencies, in the range of 10^{-3}, and obtain stable clones of insertional mutants in a few weeks instead of 6–12 months. Our results show that the piggyBac transposition system can be used as an efficient, random integration tool needed for large-scale, whole-genome mutagenesis of malaria parasites. The availability of such an adaptable genetic tool opens the way for much needed forward genetic approaches to study this lethal human parasite.

Transposable elements have been widely used as tools to manipulate genomes ranging from different microbes to higher invertebrates like *Drosophila*, mammals, and even plants. Transposable elements do not occur naturally in many lower eukaryotes, including *Plasmodium* (12). So far, efficient transposon-mediated random mutagenesis in parasitic protozoa has been reported only in *Leishmania* (13). There has been one report of transposition in *Plasmodium* using the *Drosophila mariner* transposable element, but the transposition events occurred at a very low frequency independent of transposase and with only two integrations in the same locus (14).

The piggyBac transposable element is derived from the cabbage looper moth *Trichoplusia ni* and is a member of the TTAA-target site-specific class of transposable elements (15–18). piggyBac is a class II transposable element that exclusively targets the tetra nucleotide target site TTAA and always inserts and excises in a precise manner. piggyBac-based transposon vectors have been widely used to manipulate genomes of various invertebrate species, and piggyBac is currently the preferred vector of choice for enhancer trapping, gene discovery, and identifying gene function in *Drosophila*, other insects, and mammals (19–24). The attribute of piggyBac to nonpreferentially integrate into the genome of *Drosophila* has made it more attractive than the popular P-element, which seems to have preferential hot spots for insertion in untranslated 5′ regulatory sequences (20). In this report, we tested piggyBac’s ability to integrate into the *P. falciparum* genome to assess its potential for use as a high-throughput tool to manipulate a genetic system that has been otherwise difficult to manipulate.

**Methods**

**Genomic DNA Extraction.** *P. falciparum* genomic DNA was isolated from blood-stage parasites by using a standard phenol/chloroform method. Briefly, parasite cultures were lysed in 0.15% saponin in TSE (50 mM Tris, pH 8.0/50 mM EDTA/100 mM NaCl), incubated for 30 min at 37°C, and washed twice in TSE. The DNA-containing pellet was resuspended in TSE containing 2% SDS and proteinase K (100 μg/ml) and incubated at 37°C overnight. Extraction was performed twice in phenol/chloroform (1:1) and once in chloroform. DNA was precipitated from the aqueous phase with 3 M sodium acetate (1/10 vol) and ice-cold 100% ethanol (2 vol), allowed to incubate (5 min at −20°C), centrifuged (12,000 × g with no brake), and washed twice with 70% ethanol. The pellet was air-dried and resuspended in TE (10 mM Tris-HCl, pH 8.0/1 mM EDTA).

**PCR Amplification and Cloning.** A 1.8-kb fragment 5′ to the coding sequence of hsp 86 was amplified from *P. falciparum* 3D7 clone

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2. Abbreviations: hdhfr, human dihydrofolate reductase; ITR, inverted terminal repeat.

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by using primers 5'-taataagtacaggtagatatatttatasatatagatgat-3' and 5'-tccgtatgctattaattttcc-gaagttgt-3', the PCRs using standard reagents were subjected to 35 cycles of 15 s at 94°C, 30 s at 45°C, and 2 min at 65°C. A 1-kb fragment 3' to the coding sequence of hsp 86 was amplified from *P. falciparum* 3D7 by using primers 5'-ataaggctgagttatatataataatattatag-3' and 5'-ataaggctATTAGGAAACAAAAATGAAAG-3', and the PCR conditions were 35 cycles of 15 s at 94°C, 30 s at 41°C, and 1 min at 65°C. Both PCR products were then cloned into the vector pCR2.1 by using the TOPO TA-cloning kit (Invitrogen).

**Plasmid Constructs.** pXL-BACII-DHFR contained the human dihydrofolate reductase (*hdhfr*) coding sequence under the control of 5' *calmodulin* and 3' *histidine-rich protein-2* was excised as a 2.2-kb BglIII-EcoRI fragment from the plasmid pH11 (25) and cloned between the inverted terminal repeats of the piggyBac element in the vector pXL-BacII (26).

pHTH, the piggyBac transposase coding sequence, was cut from the vector p32 as a BamHI fragment, cloned downstream of 5' *hsp86* in pCR2.1, and screened for the right orientation. 3' *hsp86* was cut out as a HindIII fragment from pCR2.1, cloned downstream to the transposase coding sequence, and screened for the right orientation to complete the helper plasmid.

**Parasite Culture, Transfection, and Selection of Transformed Parasites.** The NF54 clone of *P. falciparum* was obtained from the Naval Medical Research Center and maintained in culture according to standard methods at 37°C and gassing (5% O₂ and 5% CO₂, nitrogen balanced) with 5% hematocrit in RPMI medium 1640 supplemented with 0.5% AlbuMax I (Invitrogen), 0.25% sodium bicarbonate, and 0.01 mg/ml gentamicin. Human RBCs were obtained from the Indiana Blood Bank and were washed three times with RPMI medium 1640, resuspended to 50% hematocrit, and stored at 4°C.

Transfection of *P. falciparum* NF54 was achieved by parasite invasion of plasmid DNA-loaded RBCs (27). For plasmid DNA loading, 250 µl of processed RBCs were washed once with 250 µl of incomplete cytominx and combined with the desired amount of plasmid DNA and incomplete cytominx to a final volume of 400 µl. The mixture was transferred to a 0.2-cm cuvette (Bio-Rad), chilled on ice, and electroporated by using a Bio-Rad Gene Pulser II and standard conditions of 0.31 kV and 950 µF capacitance. The RBCs were loaded with either 100 µg each of plasmids pXL-BacII-DHFR and pHTH (experiment 1) or with 100 µg of pXL-BacII-DHFR and 50 µg of pHTH (experiments 2–8).

To ensure parasite invasion of only loaded RBCs, late-stage parasites were purified from culture by passage through a MACS magnetic column (Miltenyi Biotec). In brief, 20 ml of parasite culture at ~5–10% late-stage parasitemia was passed through the MACS column with a magnet attached. The column was washed with 30 ml of incomplete RPMI medium 1640 to remove uninfected RBCs and early-stage parasites. The column was removed from the magnet, and the late-stage parasites still bound to the column were eluted in 20 ml of incomplete RPMI medium 1640 and pelleted by centrifugation at 1,250 × g for 5 min without brakes. The pellet was washed once with complete media and resuspended in 1 ml of complete media. In experiments 1 and 2, transfections were initiated in a 5-ml culture volume with 2 × 10⁸ parasites, and, after 48-h posttransfection, 2.5 nM WR99210 was added to the culture, and the parasites were maintained in the drug until the reappearance of parasites in Giemsa-stained smears. For experiments 3–6, parasites were initiated in a 200-µl culture volume of a 96-well microtiter plate with 2 × 10³ parasites. Experiments 7 and 8 were initiated in a 5-ml culture volume with 1 × 10⁹ parasites. For experiments 3–8, 2.5 nM WR99210 was added to the culture after four generations of growth. After the parasites reappeared in Giemsa-stained smears, the concentration of WR99210 was increased to 5 nM, and genomic DNA was extracted from drug-resistant parasites for Southern blot hybridizations.

**Limiting Dilution of Parasite Clones and Parasite Lactate Dehydrogenase Assay.** Drug-resistant parasites were cloned by limiting dilution at 0.5 and 0.25 parasites per well in a 96-well plate. Parasitemia was counted by Giemsa-stained smears, and parasites were diluted in RPMI medium 1640, such that there were 10 parasites per µl. For each plate, 500 parasites were mixed with 19.2 ml of RPMI medium 1640 and 0.8 ml of 50% hematocrit. 200 µl of this mixture were added to each well in a 96-well plate to obtain a final dilution of 0.5 parasite per well at 2% hematocrit. Similarly, 250 parasites were used as above to obtain a dilution of 0.25 parasite per well. The culture medium was changed and 0.4% hematocrit added on day 7 and day 14. On day 17, the presence of parasites was detected by a parasite lactate dehydrogenase assay (28). Briefly, 20 µl of parasite culture was lysed by freeze-thawing three times, and 100 µl of Malstat reagent (Flow Laboratories) was added. Ten microliters of 1 mg/ml nitroblue tetrazolium (Sigma) and 10 µl of 2 mg/ml diaphorase (Sigma) were added to the mixture. The mixture was incubated at room temperature for 20 min, and wells positive for parasites were identified by colorimetric analysis.

**Inverse PCR and Sequencing.** piggyBac insertion sites in transformed parasites were identified by using an inverse PCR technique (29). Genomic DNA (1 µg) from drug-resistant parasite populations was digested overnight with 10 units of Sau3AI or Rsal, precipitated with 2 vol of ethanol, and self-ligated in a 100-µl reaction. The ligation reaction was precipitated as above and resuspended in 20 µl of ddH₂O. The Sau3AI-digested, self-ligated fragments were digested with 10 units of TseI to remove the episomal fragments. The inverted terminal repeat (ITR) 2 insertion sites of piggyBac element were identified by using 1 µl of the Sau3AI/TseI-digested or Rsal-digested ligation reaction as a template for the inverse PCR (35 cycles of 94°C for 30 s, 45°C for 30 s, and 65°C for 2 min) with one of the sense primers 5'-AGATGTGCTTAATGTCAGAGCGACACG3', 5'-CTCCAAGGCGGCACTGAG3', or 5'-GATTGACAAAGCCGCTCCATTGAC3' and one of the antisense primers 5'-GTAATGTCAGAGCGACACG3', 5'-TACGACAGGGCACTGAG3', or 5'-GACGCGCATGATTTTCTTACGTGAC. The PCR products were cloned into pGEM T-Easy vector (Promega) and sequenced by the dyeoxy nucleotide chain termination method by using M13 forward and reverse primers in a Beckman CEQ 8000 sequencer.

**Sequence Analysis and Identification of Insertion Sites.** The sequences of piggyBac insertion sites were obtained by sequencing inverse PCR products. The sequences obtained by sequencing inverse PCR products were analyzed by using MACVECTOR software (Accelrys, San Diego), and the insertion sites in the genome were identified by performing a BLAST search using the PlasmoDB database (30).
PCR for Confirmation of ITR1 Insertion. To confirm the complete insertion of the piggyBac element into the \textit{P. falciparum} genome, locus-specific primer for each insertion was used in a PCR reaction with one of the antisense primers 5/-H11032-AGATCTCCTAAATGCACAGCGAC-3/ and 5/-H11032-CCTCGATATACAGACCGATAAAAC-3/ or 5/-H11032-GTTTGTTGAATTTATTATTAGTA TGTAAGT-3/. The locus-specific primers for the insertions were “a,” 5/-GTTTGTATGTATGTGTGTTTTC-3/; “c,” 5/-GGGAAATTAT AAAATGGATTATAGG-3/; “d,” 5/-CCTTTATGAATGCCGCAAC-3/; “e,” 5/-ATGGGATCCACCATATGTATAACC-3/; “f,” 5/- GGACGGGCTAATATCCTTACG-3/; and “i,” 5/-CTTGATGGAAAAATGATAGGATC-3/. The conditions for the PCR were 35 cycles of 94°C for 15 s, 45°C for 30 s, and 65°C for 1 min. The PCR products were cloned into the pGEM T-Easy vector and sequenced to confirm the presence of ITR1 and duplication of the target site TTAA.

**Results**

**Design of the piggyBac Transposon and Helper Plasmids for Use in \textit{P. falciparum}**. A minimal piggyBac transposon vector, pXL-BACII-DHFR, was created by cloning the \textit{hdhfr} coding sequence under the control of \textit{Plasmodium} 5’ and 3’ regulatory elements of \textit{calmodulin} and \textit{histidine-rich protein-2}, respectively, in the plasmid vector pXL-BACII (26). This drug-resistance cassette, widely used for malaria parasite transformation by targeted homologous recombination, was flanked by the 3’ ITR1 and the 5’ ITR2 of the piggyBac element (Fig. 1A). The ITRs are oriented such that, upon transposition, they will carry the drug-resistant cassette into the \textit{Plasmodium} genome without any of the plasmid backbone. A helper plasmid, pHTH, was created by cloning the piggyBac transposase coding sequence under the control of \textit{P. falciparum} 5’ and 3’ hsp 86 regulatory elements to mobilize the piggyBac element in the erythrocytic stages of \textit{P. falciparum} (Fig. 1B). Intended only for transient transfection, this helper plasmid contained no selectable marker.

**piggyBac Transformation of \textit{P. falciparum} Is Rapid and Efficient.** Mature blood-stage parasites were purified by isolation on a magnetic column (Miltenyi Biotec) and reintroduced into culture with erythrocytes preloaded by electroporation with plasmids pXL-BacII-DHFR and pHTH. After one to four generations of growth in the preloaded erythrocytes, parasites were selected with 2.5 nM WR99210 until drug-resistant parasites emerged in culture.
parasites were grown in 5-ml culture volume for four generations before drug selection. In protocol C (experiments 3–6), parasites were grown in a 96-well plate in a low culture volume for four generations before drug selection. Southern blot hybridizations were performed on the drug-resistant populations obtained from these eight different experiments by using a hdhfr probe. Novel hybridization bands were detected in the parasite populations, in addition to the episomal band, indicating multiple unique integrations of the piggyBac element into the genome (Fig. 2A). The average transformation efficiency of piggyBac was highest for protocol C, in the range of $10^{-3}$ (Table 1). As expected, there was no evidence for piggyBac insertions in the absence of the helper plasmid (data not shown).

**Insertions Are Stable.** To test the stability of piggyBac integrations in the genome, parasites from populations 1 and 2 were cloned by a limiting-dilution method (32). Southern blot hybridizations with a hdhfr probe identified clones with integrations into the genome. Clones A1, B8, B12, C8, and F4, derived from population 1, appeared to have the same integration “a,” whereas clones B4 and G5, derived from population 2, had two different integrations, “b” and “c” (Fig. 2B). These clones were maintained in culture for $>20$ generations in the absence of the helper plasmid. The integrated piggyBac cassette was stable in all of the clones (data not shown).

**piggyBac Integrations Are TTAA-Site-Specific.** The sites of integration in the transformed populations were identified by inverse PCR analyses performed using inverted oligonucleotide primers within the ITR2 arm of the transposed piggyBac element (29). From the multiple integrations obtained in the transformed populations, we isolated and identified 10 different insertion sites. These insertions were identified with ease because they represented the predominant population in each transfection experiment (Fig. 2A). Sequence analysis of these 10 insertions confirmed a consensus TTAA-site-specific integration of the piggyBac element into the parasite genome, as expected for authentic transposition (Fig. 3).

Integration of the ITR1 of the piggyBac element was confirmed in separate PCR reactions using locus-specific primers and a primer in the ITR1 of piggyBac. Sequence analysis confirmed the expected TTAA duplication at the ITR1 end of the insertion for all integrations, except for integrations b, g, h, and i, because of the AT-rich repeat regions in those sequences. Instead, we confirmed the complete integration of the DHFR cassette in these populations by Southern hybridization restriction fragment length polymorphism analysis (data not shown).

Six insertions (c, f, g, h, i, and j) were in the 5' region of the closest ORFs. Insertions c, i, and j were located $\sim1,000$ bp 5' to the nearby ORF, whereas insertions f, g, and h were $\sim300$ bp, 350 bp, and 500 bp upstream, respectively. Insertions a, d, and e were 100 bp, 150 bp, and 465 bp downstream of the closest ORFs, respectively. Insertion b was $\sim100$ bp downstream of the start codon of a hypothetical asparagine-rich protein (PFD0200c), thereby disrupting the putative ORF of this gene. Further analysis is needed to characterize the effects of these insertions on gene expression in these transgenic parasite lines. The 10 identified insertions were widely dispersed on different chromosomes throughout the genome of the parasite, consistent with random selection of the TTAA-target insertion sites (Fig. 3).

**Discussion**

Genome sequencing of *P. falciparum* is almost complete, but many genes have unknown functions with no homologue in other species. Therefore, it is imperative that functional genomic studies are carried out with this important pathogen. Overcoming our limited ability to genetically manipulate this organism is vital so that progress can be made in understanding the parasite's unique biology. Sophisticated genetic procedures similar to what is avail-
Inverse PCR analysis was used to identify the piggyBac insertion sites in the P. falciparum genome. Inverse PCR analysis was used to identify the piggyBac 5’ TR insertion sites. Briefly, genomic DNA from drug-resistant populations were digested with either Sau3AI or RsaI and self-ligated in a dilute reaction. Sau3AI self-ligated fragments were digested with TseI to remove the episomal fragment. The remaining self-ligated fragments were used as templates in an inverse PCR to identify sites of integration into the genome. Sequence analysis identified nine different sites of integration in eight different chromosomes, suggesting a genome-wide insertion of piggyBac. As expected, the piggyBac element had inserted in a TATA target sequence in all of the analyzed clones. PCR analysis was then performed by using a genomic primer at each insertion site and a primer in ITR1 to confirm that the insertion of the piggyBac element was complete. Further sequence analysis confirmed the insertion of piggyBac ITRs into a TATA target sequence that resulted in the duplication of the target site in the genome. The italicized sequences in insertions b, g, h, and i were confirmed by Southern blot hybridization analyses (data not shown).

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### Fig. 3.
Identification of piggyBac integration sites in the *P. falciparum* genome. Inverse PCR analysis was used to identify the piggyBac 5’ TR insertion sites. Briefly, genomic DNA from drug-resistant populations were digested with either Sau3AI or RsaI and self-ligated in a dilute reaction. Sau3AI self-ligated fragments were digested with TseI to remove the episomal fragment. The remaining self-ligated fragments were used as templates in an inverse PCR to identify sites of integration into the genome. Sequence analysis identified nine different sites of integration in eight different chromosomes, suggesting a genome-wide insertion of piggyBac. As expected, the piggyBac element had inserted in a TATA target sequence in all of the analyzed clones. PCR analysis was then performed by using a genomic primer at each insertion site and a primer in ITR1 to confirm that the insertion of the piggyBac element was complete. Further sequence analysis confirmed the insertion of piggyBac ITRs into a TATA target sequence that resulted in the duplication of the target site in the genome.

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