Published in final edited form as:

Nat Methods. 2006 August ; 3(8): 615–621. doi:10.1038/nmeth904.

Efficient site-specific integration in *Plasmodium falciparum* chromosomes mediated by mycobacteriophage Bxb1 integrase

Louis J Nkrumah¹, Rebecca A Muhle¹, Pedro A Moura¹, Pallavi Ghosh², Graham F Hatfull^{2,3}, William R Jacobs Jr^{1,4}, and David A Fidock¹

¹ Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA

² Department of Biological Sciences, University of Pittsburgh, 4249 5th Avenue, Pittsburgh, Pennsylvania 15260, USA

³ Howard Hughes Medical Institute, University of Pittsburgh, 4249 5th Avenue, Pittsburgh, Pennsylvania 15260, USA

⁴ Howard Hughes Medical Institute, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, USA

Abstract

Here we report an efficient, site-specific system of genetic integration into *Plasmodium falciparum* malaria parasite chromosomes. This is mediated by mycobacteriophage Bxb1 integrase, which catalyzes recombination between an incoming *attP* and a chromosomal *attB* site. We developed *P. falciparum* lines with the *attB* site integrated into the glutaredoxin-like *cg6* gene. Transfection of these *attB*⁺ lines with a dual-plasmid system, expressing a transgene on an *attP*-containing plasmid together with a drug resistance gene and the integrase on a separate plasmid, produced recombinant parasites within 2 to 4 weeks that were genetically uniform for single-copy plasmid integration. Integrase-mediated recombination resulted in proper targeting of parasite proteins to intra-erythrocytic compartments, including the apicoplast, a plastid-like organelle. Recombinant *attB* × *attP* parasites were genetically stable in the absence of drug and were phenotypically homogeneous. This system can be exploited for rapid genetic integration and complementation analyses at any stage of the *P. falciparum* life cycle, and it illustrates the utility of Bxb1-based integrative recombination for genetic studies of intracellular eukaryotic organisms.

In response to the worsening burden of *Plasmodium falciparum* malaria, intense research efforts have led to the development of various tools for the genetic manipulation of this organism. The application of techniques such as gene disruption and allelic replacement has afforded important insights into parasite mechanisms of drug resistance, pathogenesis, host cell invasion and transmission¹. Yet genomic integration studies are plagued by low transfection and recombination efficiencies. In addition, transgene expression assays have been

Correspondence should be addressed to D.A.F. (dfidock@aecom.yu.edu).

Note: Supplementary information is available on the Nature Methods website.

AUTHOR CONTRIBUTIONS

L.J.N., R.A.M., P.A.M., W.R.J. and D.A.F. designed the experiments, which were performed by L.J.N., R.A.M. and P.A.M. P.G. and G.F.H. provided integrase reagents and W.R.J., G.F.H. and D.A.F. conceived of the plan to test Bxb1 integrase in *P. falciparum*. All authors contributed to the manuscript, which was written primarily by L.J.N., R.A.M. and D.A.F. L.J.N. was co-mentored by D.A.F. and W.R.J.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

restricted to episomally replicating plasmids that parse unequally during mitotic segregation and hence cannot be selected in sexual stages and propagated through the life cycle¹.

Integrative recombination offers a powerful approach to target foreign DNA into specific genomic locations. Bacteriophage serine integrase-based systems are particularly suitable because of their intrinsically high efficiency of recombination between host *attB* and phage *attP* sites, with integration and excision having distinct reaction requirements that preclude spontaneous reversion events². These recombinases, unlike their tyrosine counterparts such as lambda integrase (used to develop the Invitrogen GATEWAY site-specific recombination system), are sufficient to catalyze recombination and do not require bacterial host factors. Among the serine integrases, the most extensively used has been *Streptomyces* sp. phage phiC31, which efficiently integrates plasmid vectors into prokaryotic or eukaryotic hosts containing *att* or pseudo-*att* sites^{2,3}.

The serine integrase of the mycobacteriophage Bxb1, which is a temperate phage whose genome recombines into an *attB* site located within the *Mycobacterium smegmatis* *groEL1* gene⁴, has been used to catalyze recombination in *M. smegmatis*, in *Escherichia coli*^{5,6} and *in vitro*. Recombination occurs between short *attB* and *attP* substrates, each of which contain regions of inverted symmetry flanking a conserved 5' GT dinucleotide, whose nonpalindromic nature determines the polarity of integration. Both Bxb1 and phiC31 integrases mediate integrative recombination without a requirement for divalent cations, cofactors or DNA supercoiling, making them ideally suited for targeted gene delivery^{2,5}.

In this study, we describe the development of a new means of genetic integration into *P. falciparum*. In this system, transient expression of the mycobacteriophage Bxb1 integrase mediates recombination between an *attP*-bearing plasmid and an *attB* site integrated into the *P. falciparum* genome. Using a dual-plasmid approach, we achieved rapid site-specific integration of single-copy plasmids that properly targeted proteins to intracellular parasite compartments. Integrase-mediated recombination produced genetically and phenotypically homogeneous populations of transgenic parasites within 1 month, and these remained stable in the absence of drug.

RESULTS

Generation of *attB*⁺ recombinant *P. falciparum*

We introduced the *attB* site (from *M. smegmatis*) into the *P. falciparum* *cg6* gene⁷, the disruption of which is not detrimental for asexual blood stage growth (D.F., unpublished data). To achieve this, we transfected Dd2 and 3D7 parasite strains with a plasmid (pCG6-*attB*) containing a 40-bp *attB* sequence, a truncated *cg6* insert and the human dihydrofolate reductase (*DHFR*) selectable marker (Fig. 1a and Supplementary Fig. 1 online). We selected transfected parasites with WR99210 (a specific inhibitor of *P. falciparum* *DHFR*) and used PCR to screen for plasmid recombination into the *cg6* locus. This led to the identification of lines that had integrated the *attB* site downstream of a disrupted *cg6* lacking its 3' coding sequence and 3' untranslated region (UTR). We obtained *attB*⁺ clones, referred to as Dd2^{attB} and 3D7^{attB}, by limiting dilution (Table 1). Southern blots confirmed insertion of a single *attB* site into the *cg6* locus in these Dd2^{attB} and 3D7^{attB} clones (Fig. 1b,c). To generate the control Dd2^{C1} clone, we transfected Dd2 with the pCG6 plasmid (without *attB*) that also integrated into *cg6*. This clone served to test the site specificity and relative efficiency of Bxb1 integrase-mediated recombination. We also generated clones in Dd2 and 3D7 (denoted Dd2^{attB/hrp3} and 3D7^{attB/hrp3}) that had undergone pCG6-*attB* integration into the *hrp3* 5' UTR used to drive the human *DHFR* selectable marker, as confirmed by Southern blot analysis (Table 1 and Supplementary Fig. 2 online).

Bxb1 integrase mediates rapid *attB* × *attP* recombination

To test whether mycobacteriophage Bxb1 integrase could catalyze recombination between an *attP* plasmid and an *attB* site resident in the *P. falciparum* genome, we transfected Dd2^{attB} and 3D7^{attB} clones with a plasmid (pBSD-GFP-INT-attP) containing a 50-bp *attP* element, the integrase gene and a blasticidin *S*-deaminase (*bsd*) selectable marker fused with *GFP* (Fig. 2a and Supplementary Fig. 1). This produced the lines Dd2^{attB}/BSD-GFP and 3D7^{attB}/BSD-GFP (Table 1). We also electroporated pBSD-GFP-INT-attP into the Dd2^{C1} clone lacking *attB*, to produce the control line Dd2^{C1}/BSD-GFP. Parasites appeared in the Dd2^{attB}/BSD-GFP and 3D7^{attB}/BSD-GFP lines within 9–14 d of dual drug selection with WR99210 and blasticidin (BSD), and after a few days they attained a growth rate comparable to nontransfected parasites. In contrast, we first observed parasites in the Dd2^{C1}/BSD-GFP control line on day 25, and the parasitemia remained low until 36 d post-electroporation, when the growth rate became typical of episomally transfected lines. These observations provided initial indications that *attB* × *attP* recombination had markedly increased the transfection efficiency and decreased the time required for the appearance of transfected parasites.

PCR screening for Bxb1-specific *attB* × *attP* recombination junctions (known as *attL* and *attR*) showed integration of the *attP* plasmid into the chromosomal *cg6-attB* site within 14 d in both the Dd2^{attB}/BSD-GFP and 3D7^{attB}/BSD-GFP lines (Fig. 2b). Recombination occurred via a directional single-site crossover, resulting in insertion of the entire plasmid containing the integrase and *bsd-GFP* expression cassettes. We also transfected Dd2^{attB} and 3D7^{attB} parasites with either one plasmid containing a single *attP* element or an equal mixture of plasmids containing one to three *attP* elements. This suggested preferential integration of plasmids harboring two to three *attP* sites (Fig. 2b). Based on these results, we subsequently used plasmids bearing two tandem *attP* sites.

Southern blot analysis of the Dd2^{attB}/BSD-GFP and 3D7^{attB}/BSD-GFP lines confirmed integration of a single (non-concatameric) plasmid into the *attB* site. In these lines, the absence of episomal plasmids was indicative of a parasite population that had uniformly undergone integrative recombination (Fig. 2c). This key finding obviated the need for cloning to obtain a genetically homogeneous population of integrants. Sequence analysis of recombination junctions showed a precise crossover between *attB* and *attP* sites through the central dinucleotide (5'-GT) within the 8-bp common core sequences (Fig. 2d), as observed with Bxb1 integration into *M. smegmatis*⁵. Fluorescence microscopy of live Dd2^{attB}/BSD-GFP parasites showed strong GFP expression in the cytoplasm (Fig. 2e), indicating that the *bsd-GFP* fusion was functional both as a selectable marker and as a reporter construct. Continual expression of integrase had no adverse effect on the growth or development of recombinant parasites and did not lead to *attP* plasmid excision from the integrated locus.

An integrated transgene expression system in *P. falciparum*

Results with the pBSD-GFP-INT-attP plasmid (Fig. 2a) showed that Bxb1 integrase could efficiently and rapidly catalyze site-specific recombination in *P. falciparum*. However, the integrase and *bsd* expression cassettes left little room to introduce a third cassette without compromising plasmid stability in *E. coli*. We therefore developed a dual-plasmid cotransfection system that expressed integrase from a separate plasmid (pINT, which used the neomycin selectable marker that confers G418 resistance^{8,9}; Supplementary Fig. 1), thereby enabling larger transgenes to be introduced into a more compact *attP* plasmid. With this strategy, only the transgene expression cassette and *bsd* selectable marker are permanently incorporated into the parasite genome.

To test this system, we used the *pfenr* (*P. falciparum* enoyl acyl carrier protein (ACP) reductase) gene, the product of which has been predicted to be transported to the apicoplast and participate

in *de novo* fatty acid type II biosynthesis¹⁰. We inserted a *pfenr-GFP* fusion into an *attP* expression plasmid to form pLN-ENR-GFP (Fig. 3a and Supplementary Fig. 1). We cotransfected two separate Dd2^{attB} cultures with pLN-ENR-GFP and pINT and placed them under triple drug selection (WR99210, BSD and G418), generating two Dd2^{attB}/ENR-GFP lines (Table 1). These showed evidence of plasmid integration into the *attB* site within 18 d of electroporation, as shown by PCR and sequencing (data not shown). Upon detection of integration, we removed G418 selection pressure to cure these lines of the integrase plasmid pINT.

Southern blot analysis of genomic DNA from both Dd2^{attB}/ENR-GFP lines harvested on day 30, without prior cloning, showed pLN-ENR-GFP integration into the chromosomal *attB* site (Fig. 3b,c). Hybridization did not uncover any signs of episomes in either line within 1 month of electroporation, consistent with a predominant population of recombinant parasites. We did not observe any integration into the endogenous *pfenr* locus (Fig. 3c). Western blot analysis with GFP-specific monoclonal antibodies detected a 68-kDa protein in Dd2^{attB}/ENR-GFP parasites, as predicted (Fig. 3d). Immunofluorescence images of Dd2^{attB}/ENR-GFP parasites colocalized PfENR-GFP with the apicoplast protein ACP (Fig. 3e,f) but not with the mitochondrial marker MitoTracker Red (MTR; Fig. 3g,h). Thus, the Bxb1 integrase-mediated recombination system permitted precise targeting of a parasite protein to its host organelle.

Bxb1 integrase-mediated integration is stable

Earlier studies have shown that Bxb1 integrase-mediated *attB* × *attP* recombination produces asymmetric *attL* and *attR* sites that cannot recombine without an exogenous excision factor⁵. However, the final recombinant locus generated in the *attB* × *attP* parasites contained duplicate *cg6* sequences that could potentially recombine to excise the entire segment of exogenous DNA and restore the original *cg6* locus (Fig. 1a). To test the stability of the *attB* × *attP* locus, we propagated Dd2^{attB}/ENR-GFP parasites in the absence of drug selection, or with either BSD or WR99210 only. As a control experiment, we cultured an episomally transfected Dd2 line (referred to as Dd2/ENR-GFP and replicating the pLN-ENR-GFP plasmid; Table 1) without BSD and assayed it for the rate of plasmid loss. *cg6* probe hybridization of DNA from Dd2^{attB}/ENR-GFP parasites cultured without drug pressure for 23 d showed no change in the organization of the recombinant locus, with no evidence of DNA rearrangement (Supplementary Fig. 3 online). In contrast, the episomal pLN-ENR-GFP plasmid was rapidly lost from Dd2/ENR-GFP parasites cultured without drug selection, as shown by *cg6* probe hybridization (Supplementary Fig. 3), consistent with previous observations¹¹. After 3–4 weeks of non-selection, we placed parasite cultures back under drug pressure. Whereas the integrant Dd2^{attB}/ENR-GFP line did not show any growth delay, the episomal Dd2/ENR-GFP line was cleared of living parasites within 3 d of reexposure to BSD (indicating loss of the plasmid-borne selectable marker). The above data demonstrate an intrinsic stability of the integrated transgene as compared with its episomal form in the absence of drug pressure.

Integrative recombination produces homogeneous parasites

We have frequently observed that episomally transfected cells express fluorescent proteins at varying intensities and that there can be a substantial subpopulation of cells that lack fluorescence altogether (data not shown, and ref. 11). We postulated that integrase-mediated insertion of a single-copy *GFP* gene into the parasite genome would result in a more homogeneous pattern of GFP fluorescence. Accordingly, we generated lines in which an *hrp3* 5' UTR-driven *GFP* transgene was expressed either episomally (the Dd2/GFP line) or from an integrated *attB* site (the Dd2^{attB}/GFP line), as confirmed by PCR analyses and plasmid rescue (Table 1). We analyzed these lines by flow cytometry to determine their GFP expression levels. We incubated cells with Hoechst 33342 and gated them for nuclear staining to separate the infected from uninfected erythrocytes (Fig. 4a). Hoechst staining of nontransfected Dd2,

episomally transfected Dd2/GFP and integrated Dd2^{attB}/GFP parasite lines showed very similar profiles that corresponded to predominantly singly infected, ring-stage parasites¹² and were distinct from uninfected red blood cells (RBCs; Fig. 4b). GFP expression profiles showed a noticeably higher percentage of GFP-expressing parasites in the integrant Dd2^{attB}/GFP line than the episomal Dd2/GFP line (94% versus 74% in this representative experiment, with the GFP-negative cells overlaying the profile observed with nontransfected Dd2). Although both transfected lines showed similar median intensities, the Dd2^{attB}/GFP integrants showed a single peak of GFP expression with minimal variance, whereas the episomally transfected Dd2/GFP line had multiple peaks of GFP fluorescence intensity with a considerably wider variance (Fig. 4b).

***attB* × *attP* transfection and integration efficiency**

We developed a 96-well assay to quantitatively compare the efficiency of transfection and integration of the Bxb1 integrase-mediated system with that of episomal transfection (Table 2). We cotransfected Dd2^{attB} parasites with the pLN-ENR-GFP and pINT plasmids or separately transfected them with the single plasmid pBSD-GFP-INT-attP. We also used these plasmids to transfect the control Dd2^{C1} clone, representing a standard episomal approach. We determined parasitemias 2 d after electroporation and plated parasites over a range of concentrations (0.63 to 10×10^5 infected RBC/well). We aliquoted each concentration into eight wells, and we detected positive wells 24 d later by their expression of *P. falciparum* lactate dehydrogenase¹³. The transfection efficiency was calculated as the total number of parasites needed to obtain a viable, transfected parasite culture (that is, two times the starting parasite numbers that yielded four positive wells out of eight).

Dd2^{attB} parasites electroporated with pBSD-GFP-INT-attP yielded one transfected parasite per 2.5×10^5 infected RBC (Table 2). Electroporation of this same plasmid into the Dd2^{C1} line required 10^6 parasites to produce the same efficiency. In the case of cotransfection with pLN-ENR-GFP and pINT, the Dd2^{attB} line yielded one transfected parasite per 1.5×10^6 infected RBC, whereas the Dd2^{C1} line yielded no parasites at any starting inoculum. We obtained comparable efficiencies from dual-plasmid transfection of Dd2^{attB} parasites and single-plasmid transfection of Dd2^{C1} parasites. PCR assays identified plasmid integration into 16/16 transfected Dd2^{attB} wells, versus 0/16 Dd2^{C1} wells (data not shown), confirming that this integrase system mediated rapid and highly efficient transgene integration.

DISCUSSION

This specific integration system, which we demonstrate is applicable to the intracellular eukaryotic organism *P. falciparum*, has a number of attractive features that distinguish it from existing methods of *P. falciparum* transfection. First, it is now possible to rapidly and specifically target transgenes directly into a known site in the *P. falciparum* genome, thereby eliminating the need to screen multiple candidate integration sites and unnecessarily culture lines that undergo unwanted integration events. *Plasmodium* is particularly suitable as a host genus for Bxb1 serine integrase-based recombination, as its AT-rich genomes have no sequences that bear any detectable homology to the GC-rich *attB* or *attP* sites. The site specificity inherent to serine integrases is distinct from the *piggyBac* and mini-Tn5 transposon systems, recently adapted to *Plasmodium*, which exploit the existence of frequently occurring transposition target sites in the parasite genome and offer promise for random mutagenesis, gene delivery or systematic gene knockout studies^{14,15}.

Second, recombination between the non-identical *attB* and *attP* sites produces asymmetric *attL* and *attR* sites that are refractory to excision by Bxb1 integrase⁵. The stability of the *attB* × *attP* locus, well-documented with the ϕ C31 integrase system in bacteria and yeast^{16,17}, was evidenced here by the lack of rearrangement or reversion to the wild-type locus in

recombinant parasites cultured without drug selection (Supplementary Fig. 3). Plasmid excision, leading to reconstitution of the *attB* locus, may nevertheless be desirable to confirm a transgene phenotype or to recycle markers. This may become feasible with the recent discovery that the Bxb1 *gp47* gene product can interact with *attL* and *attR* to mediate excision¹⁸. The stability of Bxb1 integrase-mediated integration stands in marked contrast to tyrosine-based recombinases such as *E. coli* P1 phage Cre or *Saccharomyces cerevisiae* FLP (which target *lox* or *frt* sites, respectively). These recombinases mediate reversible integration and excision events between two identical target sites but thermodynamically favor the latter and are therefore more often used to delete a genomic locus¹⁹. Of note, the FLP/*frt* system was recently developed as a tool for conditional mutagenesis in *P. berghei*²⁰.

Third, transgenes can now be rapidly integrated and carried throughout the life cycle, whereas transgene expression assays based on episomal replication are compromised by the rapid loss of episomes in the absence of selection (as happens, for example, when attempting to express transgenes in sexual stage parasites)^{11,21}. For this, the 3D7 strain used here should be beneficial, as it is known to be able to produce infectious gametocytes and to be transmitted through *Anopheles*²². Our studies indicate that 3D7^{attB} parasites produce mature gametocytes (data not shown), although their transmissibility has not been confirmed. In this line, the *attB* site is engineered into the *cg6* coding sequence, the disruption of which does not confer any growth disadvantage, at least for asexual blood-stage parasites. We also engineered an *attB* site into the *hrp3* 5' UTR, which does not disrupt the coding sequence and thus can be expected to have no adverse effect on parasite progression through the life cycle (Supplementary Fig. 2). Even if there were an effect, parasites lacking *hrp3* are capable of self-fertilization and can complete the invertebrate and vertebrate life cycle²³.

Fourth, the systematic integration of a single plasmid copy makes gene regulation studies more comparable by ensuring similar numbers of integrated plasmids (versus the episomal assays, in which episomal copy numbers typically range from 0 to 15 (refs. 8–11)). Our flow cytometry studies, comparing integrant versus episomal parasites, confirm this by showing a greater homogeneity both in the signal strength and in the percentage of parasites that express detectable levels of protein (Fig. 4). Indeed, gene regulation and protein trafficking studies may prove to benefit the most from this new system.

We have now used the Bxb1 integration system to mediate 93 separate integration events into the *attB*⁺ parasite lines described here. In every instance, PCR analyses identified *attB* × *attP* recombination (see, for example, Figs. 2 and 3 and Supplementary Fig. 4 online). Routinely, parasites are obtained between 14–25 d post-electroporation. Nevertheless, we have infrequently observed a lengthening of these times (up to 50 d, corresponding to reduced transfection efficiencies). In such cases, we have observed that integrated parasites constitute the major population and have successfully isolated the desired integrants by limiting dilution cloning. Furthermore, for these transgene studies, the size limitation of plasmids that can be successfully integrated is not yet known. Thus far, we have integrated plasmids as large as 10.2 kb, containing 5.4 kb of transgene (Supplementary Fig. 4). Evidence from mycobacteriophage, which use this method of recombination to specifically integrate 50- to 55-kb genomes into small (40- to 50- bp) *attB* sites of the host^{2,5}, suggests that *attB* × *attP* integrative recombination will be just as efficient with larger *P. falciparum* transgenes. Cloning of large *P. falciparum* transgenes in *E. coli* is notorious for plasmid scrambling and sequence deletions²⁴ and is likely to be the more frequent obstacle.

A limitation of the integration system we describe is its dependency on the use of host cell lines engineered to harbor an integrated *attB* site. The current study established such lines in both 3D7, whose genome has been sequenced²⁵, as well as the multidrug-resistant Dd2 line. We are continuing to develop new *attB*⁺ lines and have obtained *attB* integration into a

subtelomeric *var* gene in the cytoadherent A4 line²⁶. Ultimately, the choice of line and *attB* recipient site remains subject to the limitations of traditional methods of homologous recombination to insert an *attB* site.

This system adds to a repertoire of recent genetic advances in *Plasmodium* studies, which include insertional mutagenesis mediated by the type II transposable element *piggyBac*¹⁴ and mini-Tn5-mediated shuttle transposon mutagenesis¹⁵. Regulated transgene expression has also been described in *P. falciparum*, in which a tetracycline analog is used to repress transactivated gene expression²⁷. Combining our integrase system with these other techniques offers the prospect of developing efficient methods for complementation and change or loss-of-function studies. These expanded genetic techniques should benefit studies investigating biological processes in *P. falciparum* as we strive to discover and exploit weaknesses in this ecologically highly successful and lethal pathogen.

METHODS

Parasite propagation and transfection

We propagated *P. falciparum* parasites in human RBC under 5% O₂ and transfected as previously described²⁸. We selected plasmids harboring the human *DHFR* selectable marker (pCG6-attB and pCG6), the *bsd* marker (pBSD-GFP-INT-attP and pLN-ENR-GFP) or the neomycin marker (pINT) using 2.5 nM WR99210, 2.5 µg/ml BSD or 125 µg/ml G418, respectively.

Western blot analysis

We performed western blot analysis as described²⁹. We probed membranes with monoclonal antibodies to GFP (Clontech) or polyclonal antibodies to PfERD2 (ref. ³⁰; obtained from MR4, the malaria research and reference reagent resource center), which were diluted 1:20,000 and 1:7,500, respectively. We detected signals using chemiluminescence (ECL Plus; Amersham Biosciences).

Fluorescence microscopy

For GFP imaging, live infected erythrocytes were adhered to polylysine-coated MatTek glass-bottom Petri dishes. We captured images on an Olympus IX81 electronically motorized inverted microscope at 90× magnification. We performed immunofluorescence microscopy as described³¹. We used antibodies to ACP³² at a 1:500 dilution and used 1 µg/ml Hoechst 33342 (Sigma) for nuclear staining. We deposited cells on coverslips coated with 1% polyethylenimine (Sigma) and mounted them on glass slides. We captured images on an Olympus IX71 upright microscope at 100× magnification. Microscopes were equipped with 12-bit Cooke Sensicam QE air-cooled CCD cameras and were operated by IP Lab Spectrum 3.6.1 at the Analytical Imaging Facility of the Albert Einstein College of Medicine.

We stained mitochondria by treating live *pfenr-GFP* expressing parasites with 50 nM MitoTracker Red CM-H₂XRos (Molecular Probes) for 20 min at 37 °C, followed by cell fixation and Hoechst 33342 staining as described above. We imaged cells using differential interference contrast.

Flow cytometry

We incubated sorbitol doubly synchronized, late–ring stage parasites (harvested 16–20 h post-invasion) with 6.4 µg/ml Hoechst 33342 for 10 min at 37 °C. After washing in 1× PBS, we immediately analyzed cells for both GFP and Hoechst fluorescence (by exciting with a 488-nm or 350-nm laser and detecting with a 530/30 or 440/40 band pass filter, respectively) using an LSR-II flow cytometer (BD Biosciences). After compensation of the fluorescent signals,

we collected one million events from each parasite line on three separate occasions over 6 weeks. We displayed data on bi-exponential plots to represent the total Hoechst- and GFP-negative cell populations, and analyzed the data using FlowJo 8 software (Tree Star).

Additional methods

Information about plasmids and DNA analyses are available in Supplementary Methods online. A list of oligonucleotides used in this study is available in Supplementary Table 1 online.

Accession codes

pCG6-attB, pBSD-GFP-INT-attP, pLN-ENR-GFP and pINT plasmid details have been deposited into GenBank (accession numbers DQ813651–DQ813654). PlasmDB: *cg6*, PF07_0036; *hrp3*, MAL13P1.480.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank P. Wang and J. Hyde (University of Manchester, UK) for their gift of the pPKDSneoII plasmid and G. McFadden (University of Melbourne, Australia) for his kind gift of the antibodies to ACP. We also gratefully acknowledge the Burroughs Wellcome Fund for providing a New Initiatives in Malaria Research Award (to W.R.J. and D.A.F.) that provided funding for this work, as well as US National Institutes of Health grants AI59114 to G.F.H. and AI060342 to D.A.F. and W.R.J. (P01; principal investigator, James C. Sacchettini).

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Figure 1.

Introduction of *attB* into the *P. falciparum* genome. **(a)** Schematic of pCG6-*attB* integration into the *cg6* locus via homologous recombination and single-site crossover. This generated a truncated *cg6* 5' fragment, a 40-bp *attB* docking site, the human *DHFR* selectable marker (regulated by *hrp3* 5' UTR and *hrp2* 3' UTR elements), the pBS plasmid backbone and a truncated *cg6* 3' remnant. Primers p1 + p2 detected integration. Restriction digest fragments, and *cg6* and pBS probe locations, are indicated. **(b,c)** Southern blot analysis of DNA digested with *EcoRI* (E) and *BglIII* (B) and hybridized with **(b)** *cg6* or **(c)** pBS probes, respectively. Lanes 1 and 2: Dd2^{attB} clones; lane 3: Dd2^{C1} (control clone lacking *attB*); lane 4: Dd2; lanes 5 and 6: 3D7^{attB} clones; lane 7: 3D7; lane 8: pCG6-*attB*. Upon *EcoRI* and *BglIII* digestion and *cg6* hybridization, we detected bands of 4.4, 3.2 and 1.3 kb in all recombinant clones, as opposed to the 4.4- and 1.3-kb bands in Dd2 and 3D7. pBS hybridization detected a 3.2-kb band exclusively in the recombinant clones and plasmid. Additional hybridizations of *EcoRV*-digested genomic DNA and plasmid rescue experiments confirmed integration of a single plasmid copy and a lack of episomes in the recombinant clones (Fig. 3b and Supplementary Fig. 3).

Figure 2.

attB × *attP* recombination catalyzed in *cis* by integrase. (a) Schematic of integrase (*Int*)-mediated pBSD-GFP-INT-attP insertion into the *cg6-attB* locus, involving a precise, irreversible single crossover between the incoming *attP* and chromosomal *attB* sites. This produced non-identical *attL* and *attR* sites that flank the *bsd-GFP* and integrase expression cassettes (regulated by elements from calmodulin 5' UTR and *hsp86* 3' UTR, and from *PcDT* 5' UTR and *hrp2* 3' UTR, respectively). *EcoRV* (EV) and *XmaI* (X) restriction fragments and *cg6* or *bsd* probes are shown. (b) PCR screens with primers p2 + p3. Lanes 1–3: Dd2^{attB}/BSD-GFP recombinant parasites generated by transfecting Dd2^{attB} with pBSD-GFP-INT-attP plasmids containing either one to three tandem *attP* copies (lanes 1 and 2) or a single *attP* copy (lane 3); lanes 4–6: same as lanes 1–3, but using 3D7^{attB}/BSD-GFP recombinant parasites. Lane M: 1-kb Plus ladder (Invitrogen). (c) Southern blot of *EcoRV* and *XmaI*-digested DNA hybridized with *cg6* or *bsd*. Lane 1: Dd2^{attB}/BSD-GFP; lane 2: 3D7^{attB}/BSD-GFP; lane 3: Dd2^{attB}; lane 4: Dd2; lane 5: linearized pBSD-GFP-INT-attP. *cg6* hybridization produced 10.8- and 3.7-kb bands in Dd2^{attB}/BSD-GFP and 3D7^{attB}/BSD-GFP, 9.8- and 3.7-kb bands in Dd2^{attB} and a 7.1-kb band in Dd2. *bsd* hybridization identified a 10.8-kb band exclusively in Dd2^{attB}/BSD-GFP and 3D7^{attB}/BSD. (d) Sequence results of PCR products from (b), confirming integrase-mediated *attB* × *attP* strand exchange around the conserved 8-bp core sequence (bold). (e) Fluorescence microscopy of Dd2^{attB}/BSD-GFP parasites, showing BSD-GFP expression in the parasite cytoplasm surrounding the hemozoin pigment-containing digestive vacuole of a mature trophozoite. Scale bar, 5 μm.

Figure 3.

pfenr-GFP site-specific integration catalyzed in *trans* by integrase. **(a)** Schematic of our dual-plasmid, integrase-mediated *attB* × *attP* recombination approach. We cotransfected Dd2^{attB} parasites with pINT and pLN-ENR-GFP and selected with 2.5 μg/ml BSD, 2.5 nM WR99210 and 125 μg/ml G418. Integrase expression from pINT catalyzed integrative recombination of pLN-ENR-GFP into the *cg6*-*attB* site. *EcoRV* (EV) digest sizes are shown. We used PCR primers p2 + p4 to confirm pLN-ENR-GFP integration into *attB* (data not shown). **(b,c)** Southern blot of *EcoRV*-digested DNA hybridized with *cg6* or *pfenr* probes. Lanes 1 and 2: Dd2^{attB}/ENR-GFP lines; lane 3: Dd2^{attB}; lane 4: Dd2; lane 5: pLN-ENR-GFP. **(b)** *cg6* hybridization demonstrated 16.1- and 5.9-kb bands in the Dd2^{attB}/ENR-GFP lines, and 13.5- and 7.1-kb bands in Dd2^{attB} and Dd2, respectively. **(c)** *pfenr* hybridization located a 16.1-kb band in the Dd2^{attB}/ENR-GFP lines, in addition to the 6.7 kb endogenous *pfenr* band also present in Dd2^{attB} and Dd2. This probe also hybridized to the linear 8.4-kb pLN-ENR-GFP plasmid in the control plasmid lane. **(d)** Duplicate western blots of parasite protein extracts probed with antibodies to GFP or to PfERD2 (ref. 30). Lanes 1 and 2: Dd2^{attB}/ENR-GFP lines; lane 3: Dd2^{attB}; lane 4: Dd2. **(e-h)** Fluorescence microscopy of Dd2^{attB}/ENR-GFP **(e,g)** trophozoites and **(f,h)** schizonts. We incubated cells in **e** and **f** with antibodies to ACP and cells in **g** and **h** with MitoTracker Red (MTR) to localize the apicoplast and mitochondria, respectively. DIC, differential interference contrast, HT, Hoescht 33342 nuclear stain. Scale bar, 5 μm.

Figure 4.

Phenotypic homogeneity of GFP-expressing integrant parasites. **(a)** Expression of GFP from an integrated locus was more homogeneous than GFP expression from episomes, as determined by flow cytometry. We stained parasites with Hoechst 33342 dye and analyzed them by flow cytometry at 16–20 h post-invasion. Dot plots **(a)** depict Hoechst fluorescence intensity on the *x* axis and GFP fluorescence intensity on the *y* axis. Uninfected RBC showed no GFP or Hoechst fluorescence, whereas Dd2 parasites gave positive Hoechst signals but were GFP-negative. **(b)** We gated cells for positive Hoechst staining and overlaid Hoechst or GFP intensities. The left side demonstrates the very similar developmental stages of the different lines, reflecting synchronized ring-stage cultures, whereas the right side depicts differences in GFP expression in the episomal Dd2/GFP versus integrant Dd2^{attB}/GFP lines. GFP fluorescence was evident by the nearly two-log shift in peaks in the Dd2/GFP and the Dd2^{attB}/GFP lines compared to the Dd2 line. Although the median fluorescence was comparable, the Dd2/GFP line had eight visible peaks, whereas the Dd2^{attB}/GFP line contained a single peak.

Table 1

P. falciparum lines generated for the current study

Lines	Parental strain	Transfection plasmid	Plasmid integrated	<i>attB</i> docking site (locus)	<i>attB</i> × <i>attP</i> recombination	Selectable marker	Expressed transgene
Dd2 ^{antB}	Dd2	pCG6-attB	Yes	Yes (<i>cg6</i>)	No	DHFR	NA
3D7 ^{antB}	3D7	pCG6-attB	Yes	Yes (<i>cg6</i>)	No	DHFR	NA
Dd2 ^{C1}	Dd2	pCG6	Yes	No	No	DHFR	NA
Dd2 ^{antB/hrp3}	Dd2	pCG6-attB	Yes	Yes (<i>hrp3</i>)	No	DHFR	NA
3D7 ^{antB/hrp3}	3D7	pCG6-attB	Yes	Yes (<i>hrp3</i>)	No	DHFR	NA
Dd2 ^{antB} /BSD-GFP	Dd2 ^{antB}	pBSD-GFP-INT-attP	Yes	Yes (<i>cg6</i>)	Yes	DHFR, <i>bsd</i>	<i>bsd-GFP</i>
D7 ^{antB} /BSD-GFP	3D7 ^{antB}	pBSD-GFP-INT-attP	Yes	Yes (<i>cg6</i>)	Yes	DHFR, <i>bsd</i>	<i>bsd-GFP</i>
Dd2 ^{C1} /BSD-GFP	Dd2 ^{C1}	pBSD-GFP-INT-attP	No	No	No	DHFR, <i>bsd</i>	<i>bsd-GFP</i>
Dd2 ^{antB} /ENR-GFP	Dd2 ^{antB}	pLN-ENR-GFP + pINT	Yes	Yes (<i>cg6</i>)	Yes	DHFR, <i>bsd</i> , <i>Neo</i>	<i>pfeur-GFP</i>
Dd2/ENR-GFP	Dd2	pLN-ENR-GFP	No	No	No	<i>bsd</i>	<i>pfeur-GFP</i>
Dd2 ^{antB} /GFP	Dd2 ^{antB}	pLN-GFP + pINT	Yes	Yes (<i>cg6</i>)	Yes	DHFR, <i>bsd</i> , <i>Neo</i>	<i>GFP</i>
Dd2/GFP	Dd2	pLN-GFP	No	No	No	<i>bsd</i>	<i>GFP</i>

DHFR, human dihydrofolate reductase; *bsd*, blasticidin S-deaminase; *Neo*, neomycin phosphotransferase; NA, not applicable.

Table 2

Relative transfection efficiency of Bxb1 integrase-mediated integration

Plasmid(s) transfected	pBSD-GFP-INT-attP		pLN-ENR-GFP + pINT	
Parental line	Dd2 ^{attB}	Dd2 ^{C1}	Dd2 ^{attB}	Dd2 ^{C1}
Infected RBC/well ($\times 10^5$)	Number of positive wells (per replica series of 8 wells) ^{a,b}			
0.63	3	0	0	0
1.25	4	3	0	0
2.5	6	2	2	0
5.0	7	4 ^c	2	0
7.5	7	5	4	0
10	8	5	4	0

^a On day 2 post-electroporation, we plated each line in appropriate drug selection medium over six starting inocula tested in replicates of eight.

^b We detected positive wells using Malstat assays, performed on day 26 after transfection.

^c This predicts a transfection efficiency of 1.0×10^{-6} , which is very similar to earlier estimates of single-plasmid transfection efficiency in *P. falciparum*¹¹.