Towards systematic identification of *Plasmodium* essential genes by transposon shuttle mutagenesis

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ABSTRACT

After the deciphering of the genome sequences of several *Plasmodium* species, efforts must turn to elucidating gene function and identifying essential gene products. However, random approaches are lacking and gene targeting is inefficient in *Plasmodium*. Here, we established shuttle transposon mutagenesis in *Plasmodium berghei*. We constructed a mini-Tn*5* derivative that can transpose into parasite genes cloned in *Escherichia coli*, providing an efficient means of generating knockout fragments. A 10⁴-fold increase in frequencies of double-crossover homologous recombination in the parasite using a new electroporation technology permits to reproducibly generate pools of distinct mutants after transfection with mini-Tn*5*-interrupted sequences. The procedure opens the way to the systematic identification of essential genes in *Plasmodium*.

INTRODUCTION

Malaria research has entered the post-genomic era. The genome sequence of *Plasmodium falciparum* (1), the deadliest of the malaria parasites that infect humans, and of several *Plasmodium* species that infect rodents (2,3) are now known. Not surprisingly, the majority of the ~5–6000 predicted genes lack identifiable functions and appear to be unique to *Plasmodium* or to its Apicomplexa phylum. Capitalizing on the genome sequences, expression profiling techniques such as DNA microarrays (3–5) and mass spectrometry (6,7) have started to classify the genome into sets of stage-specific or temporally co-expressed genes that are potentially involved in common cellular processes during the parasite life cycle. Efficient techniques are now needed to translate sequence and expression information into biological activities.

Gene disruption is a powerful means of elucidating gene function *in vivo*. Gene targeting in the haploid *Plasmodium* is facilitated by a ~100% homologous recombination rate of transforming DNA, but remains very inefficient owing to both the difficulty in manipulating the ~80% A/T-rich *Plasmodium* DNA in *Escherichia coli* and the poor transfection frequencies in the parasite. Consequently, <50 parasite gene disruptions have been reported since the transfection technology was first established in *Plasmodium* 10 years ago (8,9), i.e. <1% of the *Plasmodium* genes. Functional approaches that do not rely on homologous recombination have not been established. Transposition into the *Plasmodium* genome was reported only once (10), using the *mariner* element, but with low efficiency incompatible with gene discovery.

Here, we report the development of a transposon-based, shuttle mutagenesis procedure in *Plasmodium berghei*. Using this method, cloned parasite genes can be interrupted by insertion of a transposon derivative in *E.coli*, and the resulting disrupted fragments can be used to replace their wild-type counterparts in the parasite genome by double-crossover (DCO) homologous recombination (Figure 1A). *P.berghei*, which infects rodents and has long been used as a model in malaria research, is a species of choice for devising more efficient molecular genetic approaches (11). *P.berghei* transfection is now well established in a number of laboratories in the world, and selection times are short. Crucial to shuttle procedures, *P.berghei* can be transfected with linear DNA and can therefore undergo DCO recombination without the need for a negative selectable marker. Finally, *P.berghei* can be cultured safely and cycled through mosquitoes routinely, thus opening the entire parasite life cycle to functional studies *in vivo*.

MATERIALS AND METHODS

Mini-Tn*5*Pb1 mutagenesis of *P.berghei* cloned DNA

The mini-Tn*5*Pb1 transposon carried by plasmid pUT was constructed by cloning the *hDHFR* selection cassette

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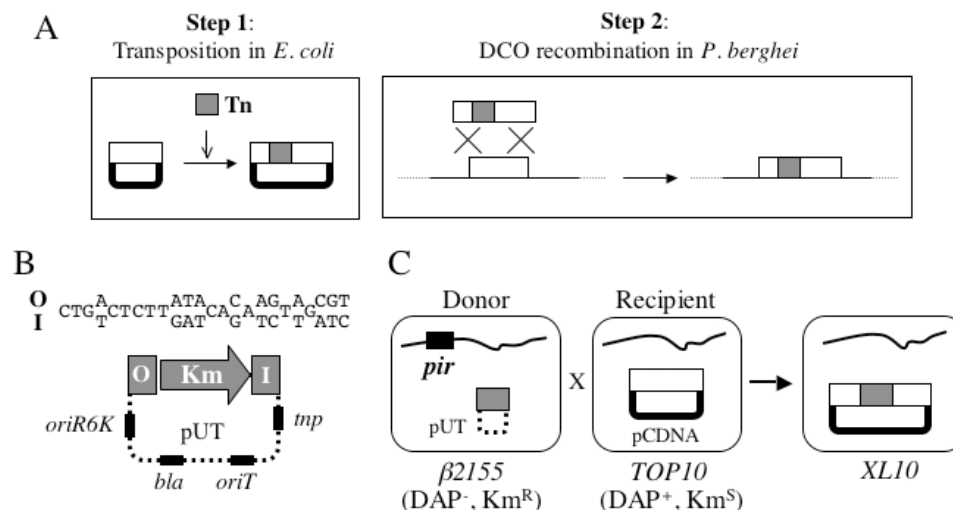
Figure 1. (**A**) The two-step, transposon shuttle mutagenesis procedure. The first step occurs in *E.coli* with insertion of a transposon (gray box) in the target DNA (open box) cloned into a plasmid (thick line). The second step occurs in *P.berghei* with DCO homologous recombination that exchanges the wild-type gene by the transposon-interrupted copy. (**B**) Genetic organization of the mini-Tn5Km2 mini-transposon and of the suicide delivery plasmid. The 1.8 kb mini-Tn5Km2 consists of a kanamycin resistance gene (Km) flanked by the two 24 bp-long terminal repeats of IS*50* of Tn*5*, called O and I ends, which are sufficient for efficient transposition of any intervening DNA. The mini-transposon is carried by the 7.1 kb-long pUT plasmid (dashed line), which is a suicide plasmid. Its *oriR6K*-dependent replication requires the π protein encoded by the *pir* gene in the donor strain, and can be mobilized to recipients via its *oriT* and RP4 mobilization functions in the donor. Plasmid pUT also contains the IS*50*$_R$ transposase *tnp* gene in *cis*, which is thus external to the mobile element, and the ampicillin-resistance *bla* gene. (**C**) The bacterial conjugation system for inserting mini-Tn5Km2 into *Plasmodium* DNA. The donor strain (β2155), which delivers the mini-Tn*5*, is resistant to kanamycin (via the plasmid-borne mini-Tn*5*) and DAP⁻ (via insertion of the *pir* gene into the chromosomal *dap* gene). The recipient strain (TOP10), which contains the target plasmid, is sensitive to kanamycin and DAP⁺. The two strains are conjugated, and transconjugants are selected on media lacking DAP (to counterselect the donor) and containing kanamycin (to counterselect the recipient). To select those transconjugants that have inserted the mini-Tn*5* in the target plasmid, plasmids are extracted from transconjugants and retransformed into *E.coli* XL10 on media containing kanamycin. Thin line, bacterial chromosome; gray box, mini-transposon; dashed line, pUT plasmid backbone; open box, target DNA; thick line, pCDNA2.1 plasmid backbone.

containing its own expression sequences (12) as a KpnI fragment into the KpnI site of mini-Tn5Km2 transposon (13). Target *P.berghei* DNA was amplified by PCR analysis from *P.berghei* NK65 genomic DNA and cloned into the pCDNA2.1 plasmid.

Transposition into *P.berghei* DNA was obtained by bacterial conjugation. The pUT plasmid containing the mini-Tn5Pb1 was transformed into strain β2155 and propagated at 37°C in 2YT liquid medium containing DAP (0.5 mM) and carbenicillin (50 μg/ml). Target constructs were transformed into strain TOP10 and propagated at 30°C on Luria–Bertani (LB) plates containing ampicillin (100 μg/ml). Transformed β2155 and TOP10 cells were cross-swabbed on LB plates supplemented with DAP (0.5 mM) and grown overnight at 37°C. Plates were scrapped to collect bacteria and the mixture was used to inoculate 2YT liquid medium containing kanamycin (150 μg/ml). Two identical flasks were prepared, in which bacteria were further grown at 37 or 30°C (in further steps, the growth temperature was kept unchanged). Growth until saturation was obtained after 16–48 h of incubation. Bacteria were then harvested by centrifugation, plasmid DNA was purified and transformed into strain XL10 Gold. Transformants were selected on plates containing kanamycin (50 μg/ml) and incubated overnight at 37 or 30°C. Isolated colonies were tested by PCR to check the presence of the mini-transposon within the cloned target DNA, using primers that hybridize to the mini-transposon or to the plasmid backbone. Selected candidates were propagated in liquid medium containing kanamycin (50 μg/ml) and the corresponding plasmid was subjected to restriction analysis.

Strain β2155 grows and conjugates better at 37°C, whereas target plasmids harboring *P.berghei* DNA are more stable when grown in *E.coli* at 30°C. Upon further kanamycin selection, exconjugants might fail to propagate at 30°C, but exconjugants selected at 37°C may suffer spontaneous deletions of the *P.berghei* insert. We therefore suggest that both 37 and 30°C growth temperatures should be used in parallel during the step of selection of the exconjugants.

After insertion of the mini-Tn5Pb1 into the target *P.berghei* DNA, and prior to electroporation into parasites, the plasmids containing the mini-Tn5Pb1-interrupted *P.berghei* fragments were digested with the ApaI restriction enzyme (GGGCCC). ApaI rarely cuts into the AT/rich *Plasmodium* DNA but cuts on both sides of the cloned fragments in the plasmid polylinker, releasing linear DCO fragments.

The purification of the DCO fragment, although not required, would reduce the risk of false-positive results, i.e. selection of episome-containing parasites. Alternatively, the presence of residual episomes in the transfected DNA can be reduced by cutting the plasmid DNA not only at both ends of the *Plasmodium* insert for liberating the DCO fragment, but also in the plasmid backbone. We have verified this by cutting the plasmids with both ApaI, which liberates the DCO fragment, and ScaI, which cuts in the plasmid backbone (*bla* gene). In this case, parasites containing episomes were not selected upon pyrimethamine treatment of electroporated parasites.

P.berghei transfection protocol

Collection of *P.berghei* parasites for transfection was essentially as described previously (14). However, parasites were

electroporated with the Nucleofector™ device (Amaxa) instead of the commonly used Gene Pulser II (Biorad). Electroporation with the Nucleofector™ device results in higher transfection efficiency (C. J. Janse, unpublished data, and Results presented here). Collection and transfection of parasites were performed as follows. After overnight parasite culture, parasites were collected at room temperature, since keeping parasites on ice was found to be deleterious to parasite survival. Prior to electroporation, 50 µl of parasites (containing $\sim 10^7/10^8$ merozoites) were mixed with 100 µl of Human T-cell Nucleofector™ solution (Amaxa) and with 5 µl of DNA (containing 1–5 µg of linear DNA in water or TE buffer). Electroporation was performed using the U33 program in the Nucleofector™ electroporator, and electroporated parasites were resuspended in 250 µl of phosphate-buffered saline (PBS) and injected intravenously 5–10 min post electroporation (pe) into 3-week-old female swiss mice. Recipient mice were treated daily with pyrimethamine (10 mg/kg) intraperitoneally (IP) for three consecutive days, starting 24 h pe. A resistant population emerged usually at day 6 pe, and was transferred to two new animals at day 6 pe, was treated with pyrimethamine (10 mg/kg) IP for three consecutive days starting 24 h after parasite transfer. The parasite resistant population usually reached a parasitemia of $\sim 1\%$ at day 10 to 12 pe.

Construction of the *TRAP* mini-library

To obtain the *TRAP* mini-library shown in Figure 4A, we first constructed plasmid pDCO-TRAP that contained (i) the 5′ part of the *TRAP* coding sequence (0.85 kb) as a left arm of homology, (ii) the 1.6 kb *hDHFR* selectable marker, (iii) a 1.2 kb DNA fragment of *Bacillus subtilis*, and (iv) the 3′ part of the *TRAP* coding sequence (0.95 kb) as the right arm of homology. Unidirectional deletions were then generated in the *B.subtilis* fragment by exonuclease III digestion. For this, an NheI restriction site (whose 3′ recessed ends are sensitive to exonuclease III) followed by a FseI restriction site (whose 5′ overhang ends are resistant to exonuclease III digestion) were inserted into the plasmid between the *B.subtilis* fragment and the *TRAP* right arm of homology. Exonuclease III was then incubated with the plasmid digested by both NheI and FseI restriction enzymes, leading to the progressive, unidirectional digestion of the plasmid. Digestion was monitored by gel electrophoresis and, when desired, the reaction was terminated, the ends of the linear molecules were flushed with S1 nuclease and polished with T4 DNA polymerase. The molecules were then recircularized with T4 DNA ligase, circular DNA was transformed into XL10 cells, and the extent of deletion in the *B.subtilis* DNA was analyzed by restriction mapping. Twenty plasmids having a *B.subtilis* DNA of different sizes (from 0.3 to 1.2 kb) that could be easily be distinguished from one another by PCR were finally selected and produced individually.

PCR analysis

Parasite genomic DNA was prepared as described previously (14). Briefly, blood from infected animals was treated with 0.2% w/v saponin, parasites were collected by centrifugation, resuspended in PBS, and incubated for 1 h at 55°C in the presence of 1% w/v SDS and 0.2 mg/ml proteinase K. The preparation was further subjected to one phenol/chloroform

extraction followed by ethanol precipitation, and the genomic DNA was collected by centrifugation and resuspended in water. The PCR was performed using polymerase *Ex Taq* (Takara) according to the manufacturer's instructions. The PCR program consisted in one step of 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, 2 min at 72°C, followed by one step of 5 min at 72°C.

RESULTS

To be a suitable approach to systematic gene disruption, the shuttle procedure needs to meet two requirements. (i) Obtaining a replacement construct should avoid *in vitro* recombinant DNA technology, which is particularly inefficient with the A/T-rich *Plasmodium* DNA, and selection in the parasite should only recover the desired DCO events. (ii) Frequencies of DCO recombination should be sufficient for creating multiple knockout (KO) parasites in a single transfection experiment, ideally allowing for phenotypical analysis of multiple mutants in a single life cycling experiment.

Mini-Tn5 mutagenesis of cloned *Plasmodium* DNA in *E.coli*

We first established transposon insertion into cloned *Plasmodium* DNA in *E.coli* for creating KO (replacement) constructs. The Tn*5* transposon is known to insert almost randomly in DNA from a wide range of bacterial species via a cut and paste process (15). Several Tn*5*-derived 'mini-transposons' have been constructed that retain transposition frequencies similar to those of Tn*5* (13), including the 1.8 kb mini-Tn*5*Km2 (Figure 1B). The mini-Tn*5*Km2 is carried by a conjugative and suicide plasmid that replicates in a π protein-producing (*pir*+) donor, but not in standard recipient strains. Insertion of mini-Tn*5*Km2 into target DNA is obtained by bacterial conjugation (Figure 1C), and appropriate selection allows for the recovery of recipients that have acquired the transposon only.

We first tested the capacity of various combinations of *E.coli* donor strains (β2155(*dap::pir*), SM10(λ*pir*), S17-1(λ*pir*)) and recipient cells (TOP10, XL10, NM554 and pop3) as mini-Tn*5*Km2 delivery systems into *Plasmodium* DNA. For this, we targeted sequences from the *CS* and *TRAP* loci of *P.berghei*. We found one highly productive combination, using β2155 as a donor and TOP10 as a recipient (Figure 1C). In β2155, the *pir* gene necessary for replication of the mini-Tn*5* delivery plasmid (pUT) is stably integrated into the *dap* gene, which renders the cell auxotroph to DAP (diaminopimelic acid, essential for bacterial wall synthesis). Therefore, exconjugants were selected on media deficient in DAP (to counterselect the donor) and containing kanamycin (to counterselect the unchanged recipient). Since the transposon can insert into the recipient's chromosome or target plasmid, the latter were recovered from exconjugants, transformed into *E.coli* XL10, and selected for with kanamycin.

Using this system, the mini-Tn*5*Km2 was found to insert with high frequency into both the *CS* and *TRAP* cloned loci (Figure 2). Up to 90–100% of kanamycin-resistant XL10 cells had the mini-Tn*5*Km2 inserted into the target plasmid, and a majority of these clones had integrated the mini-Tn*5*Km2 in the *Plasmodium* insert. Restriction analysis and sequencing of
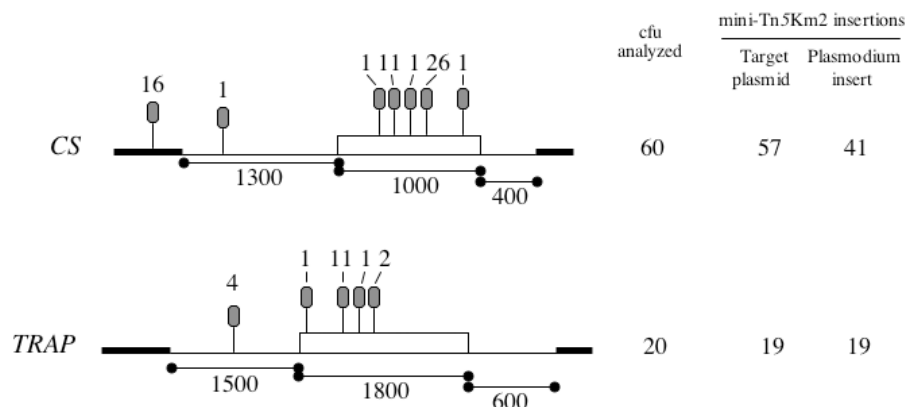
Figure 2. Mini-Tn5Km2 transposition into *P.berghei* DNA. Strain β2155 containing the mini-Tn5Km2 and strain TOP10 containing the *P.berghei CS* or *TRAP* DNA were conjugated, transformants were selected in the absence of DAP and in the presence of kanamycin, and plasmid DNA from selected colonies was analyzed by restriction mapping. In the *CS* experiment, 60 colony forming units (cfu) were analyzed and 57 contained a mini-Tn5Km2 in the target plasmid, of which 16 were located in the plasmid backbone (not mapped further) and 41 in the *CS* DNA. Of the latter, one insertion was in the *CS* 5′-untranslated DNA, whereas the remainder were in the *CS* coding sequence [at 300 bp (1), 400 bp (11), 500 bp (1), 600 bp (26), and 900 bp (1) after the *CS* start codon]. In the *TRAP* experiment, 20 cfu were analyzed, 19 of which contained a mini-Tn5Km2 in the *TRAP* DNA. Of these, 4 were located in *TRAP* 5′-untranslated DNA, whereas the remainder were in the *TRAP* coding sequence [at 150 bp (1), 650 bp (11), 750 bp (1), and 850 bp (2) after the *TRAP* start codon]. Thick line, plasmid backbone; thin line, 5′- or 3′-untranslated regions; open box, coding sequence.
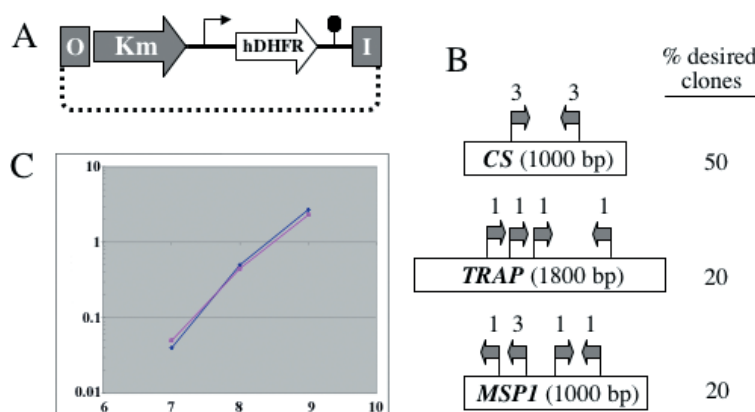

Figure 3. (**A**) Genetic organization of the mini-Tn5Pb1 mini-transposon. The 3.4 kb mini-Tn5Pb1 element is derived from mini-Tn5Km2 after insertion of the *hDHFR* marker between the kanamycin resistance gene and the I end of mini-Tn5Km2. The arrow and the ellipse symbolize *P.berghei* 5′ and 3′ expression sequences, respectively. (**B**) Mini-Tn5Pb1 transposition into *P.berghei* genes. Strain β2155 containing mini-Tn5Pb1 and strain TOP10 containing the *P.berghei* coding sequences (*CS*, *TRAP* or *MSP1*) were conjugated, and the plasmid DNA from selected transconjugants was analyzed by restriction mapping. Insertions into the coding sequences (arrows), their relative position and orientation are shown. (**C**) The mini-Tn5Pb1 does not alter *P.berghei* growth rate. Parasite growth inside rodent erythrocytes of the WT *P.berghei* NK65 (squares) and a recombinant containing the mini-Tn5Pb1 element inserted into the *TRAP* coding sequence (diamonds).

the sites of insertion revealed authentic transposition of the mini-Tn5Km2. Co-integration of the whole delivery plasmid into the target plasmid and rearrangement of the recipient DNA were not observed (data not shown). This suggested that the mini-Tn5 was suitable for mutagenesis of cloned *Plasmodium* DNA.

Construction of an *E.coli–P.berghei* shuttle mini-Tn5

For use in shuttle mutagenesis, the mini-Tn5 must also contain a marker for selection in the parasite. We used the shortest available, 1.6 kb-long pyrimethamine-resistance cassette (12), consisting of the human *DHFR* gene flanked by short *P.berghei* 5′ and 3′ expression sequences (Figure 3A). The cassette was inserted at various restriction sites in mini-Tn5Km2, and only one mini-transposon derivative had retained transposition capacity. This derivative, called

mini-Tn5Pb1, had the resistance cassette inserted 25 bases upstream from the 5′ I end of mini-Tn5Km2 (Figure 3A).

The transposition efficiency of mini-Tn5Pb1 was tested by conjugation of β2155 donor cells containing the pUT-borne mini-Tn5Pb1, and TOP10 recipient cells containing the coding sequences of the *CS*, *TRAP* or *MSP1* genes. As shown in Figure 3B, the mini-Tn5Pb1 randomly inserted into these *Plasmodium* genes. Although transposition efficiency of mini-Tn5Pb1 was lower than that of mini-Tn5Km2 (20–50% versus 90–100%, respectively), correct insertions of the mini-Tn5Pb1 into an otherwise un-rearranged *Plasmodium* insert were readily selected from kanamycin-resistant XL10 colonies. Insertions at distinct positions were obtained, and the mutated plasmid DNA could be produced in large amounts for parasite transformation.

To test whether the pyrimethamine-resistance cassette within mini-Tn5Pb1 was functional, we targeted the *TRAP*

gene, which is not required for parasite replication inside erythrocytes (16). We transformed *P.berghei* erythrocytic stages with a 1.8 kb-long *TRAP* linear fragment interrupted by mini-Tn5Pb1 (Figure 3B). Pyrimethamine treatment of mice allowed for the selection of a population of parasites that had all exchanged the *TRAP* genomic locus by the mini-Tn5Pb1-interrupted copy (data not shown). The intra-erythrocytic replication rates of the wild-type and the recombinant parasites were similar (Figure 3C), indicating that the mini-Tn5Pb1 had no adverse effect on parasite fitness. We conclude that the mini-Tn5 shuttle procedure provides an easy means of generating gene KO in *P.berghei*.

Assessing frequencies of DCO recombination in *P.berghei*

A systematic procedure of gene KO would require that the frequency of the DCO events be sufficient to create distinct KO parasites in a single transfection experiment. With current transfection protocols in *P.berghei*, however, the frequencies of transfection and of DCO recombination are low and experiments targeting a single gene occasionally fail to yield recombinant parasites. To optimize transfection efficiency and to investigate the minimal requirements for DCO recombination in *P.berghei*, we constructed a series of 20 replacement plasmids targeting the *TRAP* locus (Figure 4A) and used a new method for transfection, the Nucleofector™ technology (Amaxa). These plasmids contained identical left and right arms of homology corresponding to the 5′ and 3′ halves of the *TRAP* coding sequence, respectively. They differed by their central portion, which contained the *hDHFR* selection cassette of mini-Tn5Pb1 and a *B.subtilis* DNA sequence of gradually decreasing length, from 1200 to 300 bp. This mini-library was used to generate the 20 linear replacement fragments, which had either long (850 and 950 bp, upon EcoRI
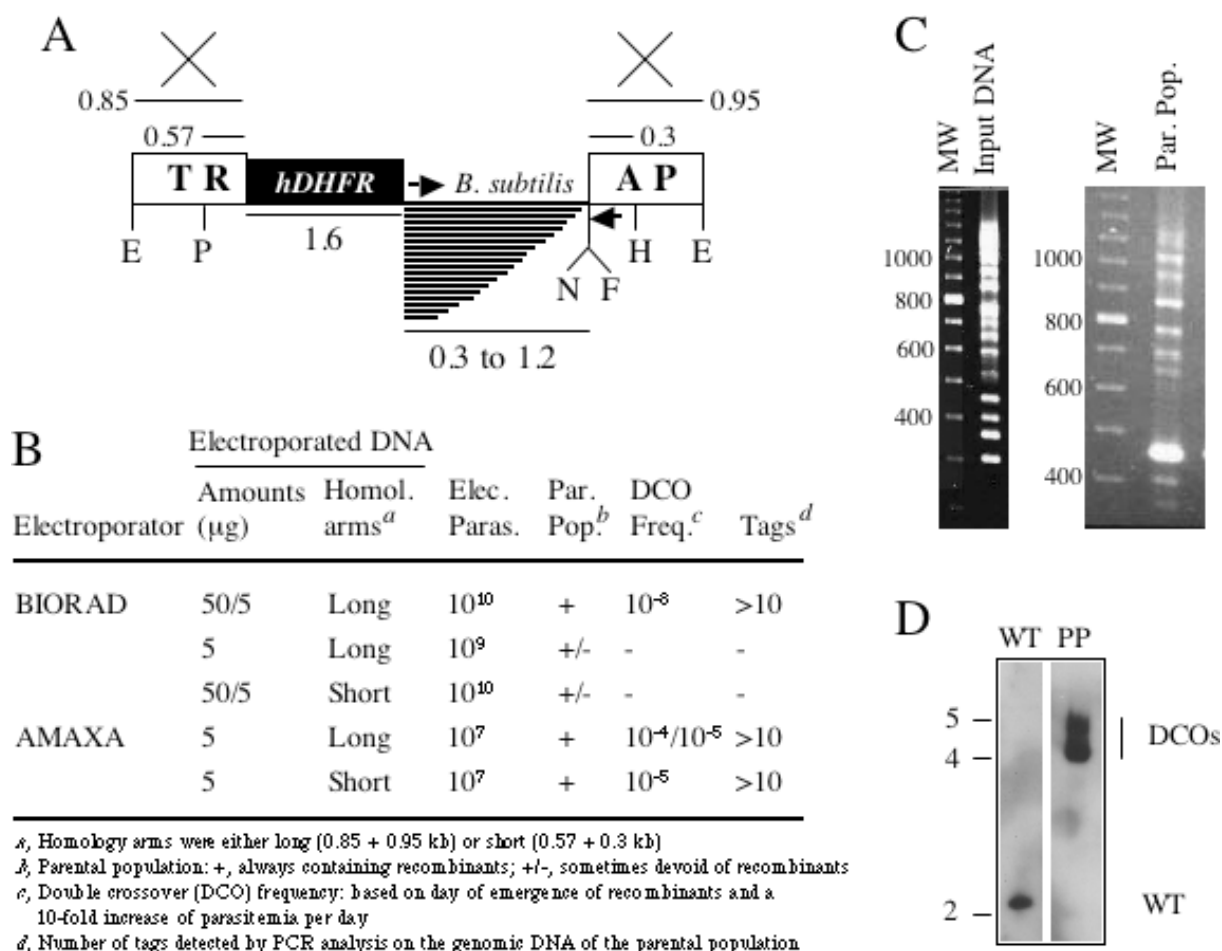

B

Electroporator	Electroporated DNA					
	Amounts (µg)	Homol. arms[a]	Elec. Paras.	Par. Pop.[b]	DCO Freq.[c]	Tags[d]
BIORAD	50/5	Long	10^{10}	+	10^{-8}	>10
	5	Long	10^{9}	+/-	-	-
	50/5	Short	10^{10}	+/-	-	-
AMAXA	5	Long	10^{7}	+	$10^{-4}/10^{-5}$	>10
	5	Short	10^{7}	+	10^{-5}	>10

a, Homology arms were either long (0.85 + 0.95 kb) or short (0.57 + 0.3 kb)
b, Parental population: +, always containing recombinants; +/-, sometimes devoid of recombinants
c, Double crossover (DCO) frequency: based on day of emergence of recombinants and a 10-fold increase of parasitemia per day
d, Number of tags detected by PCR analysis on the genomic DNA of the parental population

Figure 4. (**A**) The *TRAP* targeting mini-library. The mini-library consists of 20 replacement plasmids, which share identical *TRAP* homology arms and the *hDHFR* selectable marker, but differ by the length of a central *B.subtilis* tag (from 0.3 to 1.2 kb). The sizes of the *TRAP* homology arms after plasmid restriction digestion with EcoRI or HincII + PmlI are shown above. E, EcoRI; H, HincII; N, NheI; F, FseI; P, PmlI. (**B**) Frequencies of DCO events at the *TRAP* genomic locus. Parasites were electroporated with the *TRAP* mini-library, using various amounts and lengths of homology of the targeting DNA and the Gene Pulser II (Biorad) or the Nucleofector™ (Amaxa) electroporators. (**C**) PCR analysis of a transfected parasite population. Parasites were transfected with 50 µg of the *TRAP* mini-library having long homology arms using a Gene Pulser II (Biorad) electroporator. PCR analysis uses a forward primer hybridizing to the 5′ end of the *B.subtilis* tag and a reverse primer hybridizing to the 5′ end of the *TRAP* right homology arm (A). The PCR on the left uses the pool of transfected DNA (Input DNA) as a template, and the PCR on the right uses the genomic DNA of the resistant parental population (Par. Pop.) as a template. (**D**) Southern blot analysis of the *TRAP* locus in the wild-type and parasites transfected with the *TRAP* mini-library. Genomic DNA was cut with ClaI, which does not cut in the central region of the constructs, and hybridized with a probe corresponding to the 5′ part of the *TRAP* coding sequence. The size of the ClaI fragment in the WT (2.1 kb) increases in the parental population (DCOs) by the size of the central region of the constructs (1.9–2.8 kb).

digestion) or short (300 and 570 bp, upon HincII + PmlI digestion) arms of homology and contained the *B.subtilis* sequences of various sizes. The latter were used as tags in PCR experiments to identify the individual mutants in parasite pools.

Equal amounts of each of the 20 constructs were transfected as a pool into parasites, and the frequency of DCO was estimated based on the day of emergence of the recombinant parasites in mice and the number of distinct tags identified in these parasites by PCR analysis (Figure 4B). This system allowed us to optimize the transfection protocol (Materials and Methods). Using the Gene Pulser II (Biorad) electroporator, large numbers of parasites as well as long homology arms in the transfected DNA were necessary to reproducibly generate a population of recombinant parasites from which more than 10 distinct tags could be amplified (estimated frequency of $\sim 10^{-8}$; Figure 4B and C). Importantly, transfecting even large amounts (50 µg) of constructs with short homology arms did not ensure the reproducible selection of recombinants.

We then tested the Nucleofector™ device for electroporation. This technology requires significantly smaller numbers of parasites (Figure 4B), and populations of recombinant parasites from which more than 10 distinct tags could be amplified were reproducibly obtained. In addition, recombinant populations were obtained with higher frequencies ($10^{-4}/10^{-5}$) than when using the Gene Pulser II, regardless of the size of the homology arms in the transfected DNA. Southern blot analysis of the DNA of the selected populations also indicated that these only contained the desired recombinant parasites, with no trace of parasites having a WT *TRAP* locus or of recombinants emerging from events of non-homologous recombination (Figure 4D). These results indicate that several independent KO mutants can be generated in a single transfection experiment using the Nucleofector™ technology, even using low amounts of targeting DNA having short arms of homology (<1 kb in total).

Creating parasite mutants by shuttle transposon mutagenesis

To test whether the shuttle procedure could be used to assess gene function in a systematic fashion, we targeted, in addition to *TRAP*, the *MSP1* and *AMA1* genes, which are essential for merozoite invasion into erythrocytes (17,18), as well as the *UIS21*, *S6* and *S15* genes, which are known to be expressed in sporozoites (19,20). In each case, an internal fragment of the coding sequence was amplified from the *P.berghei* NK65 genome and was subsequently mutagenized by mini-Tn5Pb1 transposition. One replacement plasmid was selected for *MSP1*, *AMA1*, *UIS21*, *S6* and *S15*, and two for *TRAP*. These plasmids were digested by ApaI restriction digestion to liberate DCO fragments (Figure 5A) and mixed in equal amounts in a DNA pool (Figure 5B). This DNA pool was then transfected into *P.berghei* using the optimized Nucleofector™ technology, and the presence of each mutant in the selected parasite population was tested by PCR analysis using a mini-Tn5Pb1-specific primer and gene-replacement specific primers (Figure 5C; primers 1 and 3, respectively). As expected, replacement events were detected at *TRAP* in the pool of resistant parasites, but not at *MSP1* or *AMA1*. Insertions were also detected into *S6* and *S15*, but not into *UIS21*,

suggesting that the latter gene might be essential for the erythrocytic cycle.

To verify that the absence of *UIS21* mutant in the selected parasite population was indeed owing to the essential nature of UIS21, transfection experiments were repeated using *UIS21*-targeting DNA alone. First, transfection was performed with DNA digested with ApaI, which releases a DCO fragment (Figure 5D). Although a resistant parasite population was selected, PCR analysis of parasite DNA detected only the presence of *UIS21*-targeting episomes, but not that of *UIS21* gene-replacement events. Transfection was also performed with the *UIS21*-targeting plasmid linearized in the downstream region of homology at the unique BsgI site, creating a linear DNA fragment that might disrupt the endogenous *UIS21* via a single-crossover (SCO) event (Figure 5E). Again, a resistant parasite population was selected that was shown by PCR analysis to contain *UIS21*-targeting episomes, but not integration events at *UIS21*. The selection of parasites containing episomes shows that the selectable marker in the plasmid is functional, and the lack of selection of *UIS21* disruption events indicates that the protein is important for parasite multiplication in erythrocytes. We conclude that the mini-Tn5Pb1-based shuttle mutagenesis technique can be used to generate pools of *P.berghei* KO, and to identify potentially essential genes.

DISCUSSION

Forward genetic screens are required to identify *Plasmodium* genes on a functional basis, primarily those that are essential for parasite survival. Identification of essential genes should provide a starting point for uncovering new important functions in the parasite, as well as new targets for anti-malarial drugs. Screening random mutants is currently not an option, as genetic elements that insert randomly in the parasite genome have not been found or constructed. Even if technically possible, a random approach would still imply testing a large collection of mutants (many times the total number of genes) to cover the genome, which would make it particularly challenging given the complexity of the parasite life cycle through mosquitoes. However, the recent sequencing of several *Plasmodium* genomes (1–3) and their transcriptional profiling (3–5) open the way to directed, rather than random approaches to elucidating gene function. Sets of target genes can now be defined, which can then be tackled in a systematic fashion using reverse genetic tools. A complete screen would need to incorporate all the genes that are expressed during the stage/process of interest, while more focused screens might incorporate only sub-sets of these, e.g. those that are stage-specific or that encode proteins with domains of interest.

We report here a shuttle mutagenesis procedure that simplifies the construction of *P.berghei* KOs by homologous recombination. First, to create gene-replacement constructs, we established *in vivo* transposition of a shuttle mini-Tn5 derivative into parasite DNA in *E.coli*. The procedure consists of a minimum number of simple bacterial manipulations, is more efficient than the classical DNA cloning methods, provides a choice of distinct insertions, and can be automated. Second, the frequencies of parasite transfection and allelic
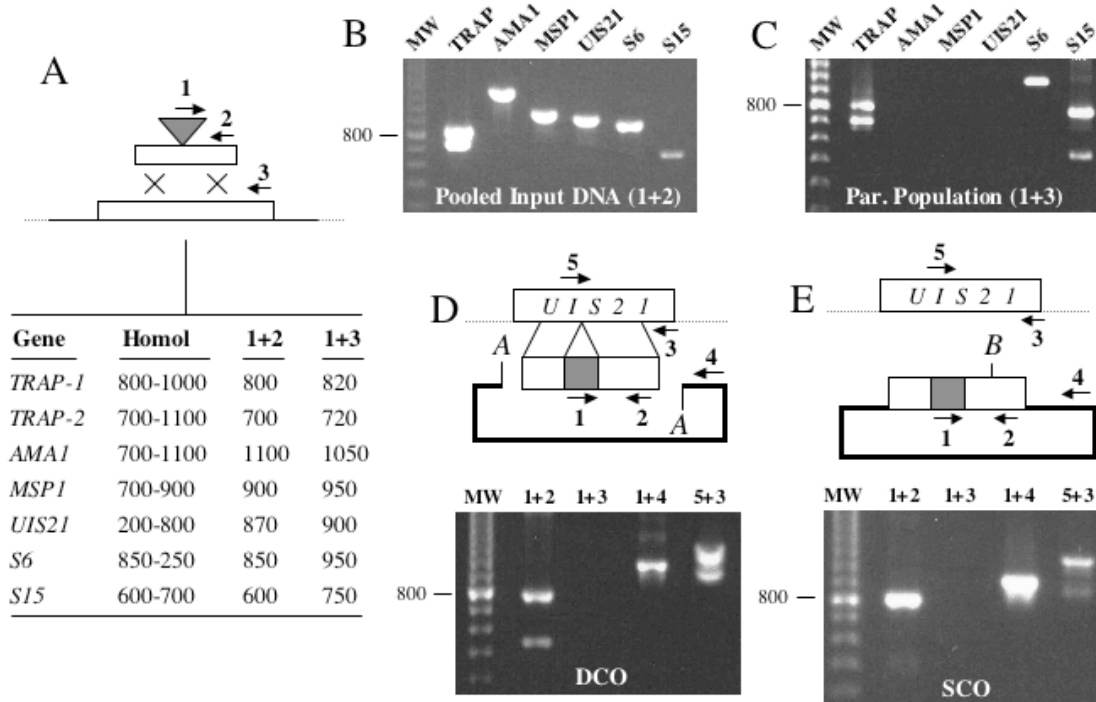
Figure 5. (**A**) Constructing a pool of mini-Tn5Pb1-interrupted *P.berghei* sequences. *P.berghei* fragments internal to genes of interest were mutagenized with the mini-Tn5Pb1 (gray triangle) and DCO constructs were selected. The genes, the sizes of the homology arms (Homol) and the expected sizes of the PCR fragments generated with primers 1, 2 and 3 [1 + 2 and 1 + 3, shown in (B and C)] are indicated. (**B**) PCR analysis of the pool of mini-Tn5Pb1-interrupted sequences. Equal amounts of each of the mini-Tn5Pb1-interrupted sequences were pooled and the DNA pool subjected to PCR analysis using a forward primer (called 1) that hybridizes to the mini-transposon and a reverse primer specific of the cloned fragment (called 2) that hybridizes to the right arm of homology [for the expected sizes of PCR fragments see (A)]. The molecular weight (MW) marker is a 100 bp-ladder, with the 800 bp band indicated. (**C**) PCR analysis of the parasite population selected after transfection with the DNA pool. The genomic DNA of the selected population was subjected to PCR analysis using forward primer 1 and a reverse, DCO-specific primer (called 3), which hybridizes in the endogenous gene downstream from the right arm of homology [for the expected sizes of PCR fragments see (A)]. (**D**) PCR analysis of the resistant parasite population selected after transfection with the *UIS21* DCO fragment. The *UIS21*-targeting DNA was digested with ApaI, which liberates the interrupted *UIS21* sequence as a linear DCO fragment, transfected into parasites, and the genomic DNA of the resistant population analyzed by PCR analysis using the primers shown. A, ApaI restriction site; open box, *UIS21* gene; gray box, mini-Tn5Pb1; thick line, plasmid backbone. (**E**) PCR analysis of the resistant parasite population selected after transfection with the *UIS21* SCO fragment. The *UIS21*-targeting DNA was digested with BsgI, which cuts once in the right arm of homology of the *UIS21*-targeting plasmid and releases 300 bp- and 500 bp-long free homology ends. The linear DNA was then transfected into parasites, and the genomic DNA of the resistant population analyzed by PCR analysis using the primers shown. B, BsgI restriction site; see (D).

exchange were increased 10^4-fold by optimization of the transfection protocol and the use of Nucleofector™ technology (Amaxa). This new transfection technique requires lower amounts of transforming DNA and of recipient parasites, and allows for DCO recombination with short arms of homology (~200 bp on one side, <1 kb total). This now permits the reproducible generation of pools of 10 and presumably more independent mutants in a single transfection experiment with a corresponding pool of mini-Tn5-interrupted sequences, without the need for DNA manipulation or parasite cloning.

Assessing multiple mutants simultaneously in a transfected parasite population (pool) should save considerable time in identifying important parasite products. As shown here, each mutant can be specifically recognized by PCR analysis using appropriate primer sets, i.e. one common primer annealing to the mini-transposon and a specific primer annealing to the target gene. At the RBC stage, the failure to select a particular mutant in the parasite pool would suggest that the corresponding gene is important for parasite replication in RBC, which could then be targeted individually for confirmation as shown here for *UIS21*. Parasite pools created at the RBC stages could then be cycled through mosquitoes, and lethal mutations screened via mutants that are lost between two subsequent

developmental stages. However, such a negative screening is only possible for haploid stages of the parasite, excluding the zygote–ookinete–oocyst stages in the mosquito that are diploid/multiploid. These stages can emerge from cross-fertilization between different mutants in the parasite pool, creating the possibility of compensation of a lethal by the wild-type allele. Once haploidy is restored (at the midgut sporozoite stage), essential genes could then be identified via the loss of the corresponding mutant from the parasite pool. For example, mutants present in the salivary gland sporozoite pool but absent in the subsequent RBC stage pool after transmission should point to genes that are important for parasite development in the mammalian liver. Negatively screening for essential genes could be further facilitated by the use mini-Tn5Pb1 derivatives carrying short molecular barcodes, which have been used extensively as clone identifiers in functional genomic studies in bacteria (21,22) and in yeast (23,24). Hybridization of pooled tags on filters or microarrays with parasite pool DNA may allow for comparing the relative abundance of each mutant in the pool. This would permit the identification not only of essential genes, via the loss of mutants in the pool, but also of any gene that contributes to parasite fitness, via the decrease in the abundance of mutants.

In comparison with *P.falciparum*, the *P.berghei* system offers the advantages of short selection times, high efficiency DCO recombination, safe parasite cycling and the potential for phenotype analysis *in vivo*. *P.berghei* should also serve as a powerful system for addressing the core biology of *Plasmodium* parasites in molecular terms. Over 80% of the *P.falciparum* genes have clear orthologs in *P.berghei*, and there is marked conservation of gene synteny within the body of each chromosome between the two species (3). However, differences do exist between the two species in the sub-telomeric regions of the chromosomes, which harbor multi-gene families involved in antigenic variation and immune evasion.

These technological advances simplify gene-targeting procedures and open new perspectives for functional genomics in *Plasmodium*. They should facilitate the functional characterization of gene clusters of interest, i.e. genes that share a common pattern of temporal expression that may suggest their involvement in a defined biological process. Because of its efficiency, the shuttle procedure should also allow us to address the 'hypothetical' portion of the parasite genome, which is likely to mediate many of the unique features of the parasite.

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