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Tricks in *Plasmodium*'s molecular repertoire - escaping 3'UTR excision-based conditional silencing of the chloroquine resistance transporter locus

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Abstract

In the human malaria parasite, *Plasmodium falciparum*, the major determinant of chloroquine resistance, *P. falciparum* chloroquine resistance transporter (*pfcr*), likely plays an essential role in asexual blood stages, thus precluding conventional gene targeting approaches. We attempted to conditionally silence the expression of its ortholog in *Plasmodium berghei* (*pbcr*) through Flp recombinase-mediated excision of the 3' untranslated region (UTR) during mosquito passage. However, parasites maintained *pbcr* expression despite 3' UTR excision. Characterization of these *pbcr* mRNAs, by 3' rapid amplification of cDNA ends, identified several replacement 3' UTR sequences. Our observations demonstrate the astounding genetic plasticity of this parasite when faced with the loss of an essential gene.

Keywords

Plasmodium berghei; *pfcr*; Sporozoite; Conditional knockdown; 3' UTR; RACE

Mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*; PlasmoDB ID: MAL7P1.27) play a central role in resistance of the human malaria parasite, *P. falciparum*, to several antimalarial drugs, most notably chloroquine (Petersen et al., 2011; Roepe, 2011; Ecker et al., 2012; Summers et al., 2012). *pfcr* encodes an integral membrane protein that localizes to the parasite's digestive vacuole, the site of hemoglobin digestion and chloroquine action. Early secondary structure predictions and homology modeling predicted that PfCRT is a transporter (Fidock et al., 2000; Martin and Kirk, 2004), and recent biochemical and pharmacological studies lend support to the hypothesis that mutant PfCRT can transport chloroquine out of the digestive vacuole (Summers and Martin, 2010; Baro et al., 2011; Sanchez et al., 2011; Griffin et al., 2012; Papakrivos et al., 2012). Nevertheless, more than a decade after its discovery, the native function of PfCRT remains

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unknown. Failed gene targeting attempts in *P. falciparum* and the mouse malaria parasite, *Plasmodium berghei*, suggest that this function is essential, at least in the parasite's asexual blood stages (Waller et al., 2003; Ecker et al., 2011). Intriguingly, PfCRT peptides have also been detected by mass spectroscopy in sporozoites (Florens et al., 2002), an extracellular stage that neither possesses a digestive vacuole nor digests hemoglobin.

To gain insight into the functional requirement for CRT across the parasite lifecycle, we made use of a conditional knockout (cKO) system recently optimized for *P. berghei* by the Ménard laboratory (Combe et al., 2009; Lacroix et al., 2011). This method is based on the Flp recombinase/Flp recognition target (Flp/*FRT*) site-specific recombination system from yeast, involving Flp recombinase-mediated recognition of two 34 bp *FRT* sites leading to excision of the intervening DNA (referred to as the *FRT*ed sequence). For *P. berghei*, this cKO approach begins by replacing the endogenous 3' untranslated region (UTR) of the targeted gene with a *FRT*ed sequence comprising a 3' UTR sequence from the thrombospondin related adhesive protein (*trap*, PlasmoDB ID: PBANKA_134980) gene followed by the downstream human dihydrofolate reductase (*dhfr*) selectable marker. This double crossover homologous recombination event is undertaken in parasite strains that harbor an integrated copy of Flp recombinase that is expressed under a mosquito stage-specific promoter such that passaging of these parasites to *Anopheles* mosquitoes initiates excision of the *FRT*ed 3' UTR. This leaves the gene of interest with no 3' UTR, thereby destabilizing transcripts and generating a functional KO. The power of this cKO system was initially demonstrated by Combe et al. (2009) who used it to silence *msp1* (essential for parasite invasion of erythrocytes) and uncovered a second role for this gene in the formation of merozoites in infected hepatocytes. More recently this system was applied to conditionally silence the parasite invasion molecules AMA1 and RON4, revealing that the former is important only for merozoite invasion of erythrocytes whereas RON4 is required for sporozoite invasion of hepatocytes (Giovannini et al., 2011). This system was also used to conditionally inactivate the *P. berghei* cGMP-dependent protein kinase (PKG), leading to the developmental arrest of late liver stage parasites and the demonstration that these stages can induce potent immunity against sporozoite challenge (Falae et al., 2010).

To conditionally silence the *P. berghei* ortholog of *pfCRT* (known as *pbCRT*; PlasmoDB ID: PBANKA_121950), we first engineered the p*PbCRT*-cKO transfection plasmid to replace the endogenous *pbCRT* 3' UTR with the *FRT*ed *trap* 3' UTR and *dhfr* marker (Fig. 1A). Briefly, the last 0.6 kb of the *pbCRT* coding sequence was PCR amplified using primers p1 + p2 (listed in Table 1); and the first 0.6 kb of *pbCRT* 3' UTR was amplified using primers p3 + p4. These fragments were cloned into p3' TRAP_ *dhfr*-*FRT* (Combe et al., 2009) via *SphI* and *NotI* (for the coding sequence) or *HindIII* and *SphI* (for the 3' UTR). Notably, our p*PbCRT*-cKO plasmid unintentionally lacked the first 12 nucleotides of the *trap* 3' UTR compared with the plasmid used by Combe et al. (2009) (see below). *SphI*-linearized plasmid was then electroporated into asexual blood stage parasites of two "deleter" strains, TRAP/FlpL or UIS4/Flp. These recipient strains express either Flp recombinase or the thermolabile version FlpL (Lacroix et al., 2011). In the TRAP/FlpL strain, recombinase expression is driven by the *trap* promoter, which becomes active in maturing oocysts and peaks in salivary gland sporozoites (Rosinski-Chupin et al., 2007). The UIS4/Flp deleter strain controls Flp expression using the *uis4* promoter, which becomes highly upregulated in salivary gland sporozoites (Matuschewski et al., 2002).

Deleter strain parasites electroporated with the p*PbCRT*-cKO plasmid were inoculated into CD-1 (Charles River) mice and subjected to two rounds of in vivo drug pressure with the parasite DHFR-specific inhibitor WR99210 (de Koning-Ward et al., 2000). This agent selects for double crossover events that have inserted the *FRT*ed *trap* 3' UTR and the WR99210-resistant human *dhfr* selectable marker into the *pbCRT* target locus (Fig. 1A).

Integrants were detected by PCR and recombinant clones were obtained by limiting dilution in mice. This cloning yielded *pbcr*t CKO-TRAP/FlpL clone 9 and *pbcr*t CKO-UIS4/FlpL clone 4, hereafter referred to as the clones T9 and U4. All procedures with CD-1 and C57BL/6J (Jackson Laboratories) mice were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Columbia University Medical Center, USA.

To test whether excision of the *FRTed* 3'UTR would occur as predicted, the pre-excision T9 and U4 clones were passaged through *Anopheles stephensi* mosquitoes. Both clones produced oocysts as well as midgut and salivary gland sporozoites at normal numbers and with apparently normal morphology (data not shown). We extracted genomic DNA from oocysts and salivary gland sporozoites (harvested from mosquitoes 20-25 days after ingestion of blood meals harboring pre-excision blood stage parasites) and assessed the *pbcr*t locus by diagnostic PCR and sequencing. PCR analysis of clone T9 is illustrated in Fig. 1B (analysis of the U4 clone yielded the same results; data not shown). These data confirmed that excision of the *FRTed* *trap* 3'UTR was occurring in midgut sporozoites and went largely to completion in salivary gland sporozoites (Fig. 1B; note that the p5 + p6 band specific for the *pbcr*t-*trap* 3'UTR locus was present in the pre-excision blood stage population but was almost absent in salivary gland sporozoites).

We also allowed T9- or U4-infected mosquitoes to feed on naïve, anesthetized C57BL/6J mice in order to test whether *pbcr*t post-excision cKO sporozoites (harboring the *pbcr*t-no3'UTR locus; Fig. 1A) were infectious. Surprisingly, both the T9 and U4 post-excision parasites were readily transmitted following sporozoite inoculation, with all mice being blood smear-positive on days 5 (T9, three out of three mice) or 7 (U4, four out of four mice; these mice were not checked on day 5). For comparison, in the same experiment two out of two mice bitten by mosquitoes infected with the parental TRAP/FlpL deleter strain (used to generate the cKO T9 parasites) were smear-positive on day 7, but not on day 5. The majority of the transmitted T9 and U4 parasites appeared to possess the *pbcr*t-no3'UTR locus, although a small signal for the non-excised *pbcr*t-*trap* 3'UTR locus was also detected by PCR (see p5 + p6 lane in post-excision bulk culture; Fig. 1B). We quantified the excision rate by SYBR Green I (Bio-Rad, USA) quantitative PCR analysis using an Opticon2 Real-Time PCR Detector (Bio-Rad). Primers p8 + p9 were directed to the *hdhfr* segment of the *FRTed* sequence, and primers p10 + p11 were directed to the reference gene *hsp70* (PlasmoDB ID: PBANKA_091440). The percent excision was then calculated based on the $\Delta\Delta C(t)$. This analysis demonstrated a $99.3 \pm 0.2\%$ and $97.1 \pm 0.1\%$ loss of the *FRTed* 3'UTR in the transmitted T9 and U4 parasites, respectively (mean $\pm$ S.D., calculated from three mice per line; each sample was run in triplicate). We note that this high degree of excision is consistent with earlier reports (Combe et al., 2009).

To obtain a pure population of *pbcr*t-no3'UTR parasites lacking a 3' regulatory sequence, we re-cloned the transmitted parasite populations by limiting dilution, yielding the post-excision blood stage clones, T9-rc and U4-rc. PCR analysis confirmed the complete absence of the *FRTed* *trap* 3'UTR (Fig. 1B; see absence of band with p5 + p6). These post-excision clones were passaged by i.v. injection into naïve mice for closer analysis of their replication fitness compared with the parental deleter strains TRAP/FlpL (for T9-rc) and UIS4/FlpL (for U4-rc). For this experiment, 10^5 parasites were injected i.v. into naïve C57BL/6J mice (five mice per line in each of two separate experiments), and monitored their parasitemias daily by flow cytometry using the DNA dye SYBR Green I and the mitochondrial vital dye MitoTracker Deep Red (Eklund et al., 2011). All four parasite lines revealed essentially the same progression of infection: a rise in parasitemia until days 5-6 peaking at ~2%, followed by a modest decline for ~3-4 days prior to resurgence of the infection (coinciding with an increase in reticulocytes towards which *P. berghei* is tropic, data not shown; Fidock et al.,

2004)). Parasitemias attained 9-12% by day 11 in all lines, confirming that all lines displayed similar growth kinetics (Fig. 2A). Given that past failed KO attempts have suggested that PfCRT and PbCRT are essential, at least in the parasite's asexual blood stages (Waller et al., 2003; Ecker et al., 2011), parasite survival following excision of the 3'UTR was surprising.

We suspected that PbCRT expression might somehow be maintained in the *pbcr*-no3'UTR parasites and extracted RNA and protein to assess whether this was indeed the case. *pbcr* mRNA was readily amplified from both post-excision clones by reverse transcriptase (RT)-PCR (results for T9-rc and the pre-excision T9 clone are shown in Fig. 2B; similar results were obtained with U4-rc and are not shown). PbCRT expression was also upheld in the post-excision T9-rc and U4-rc clones (Fig. 2C). We cannot quantify levels of protein expression due to the possible presence of truncated forms, as discussed below.

We hypothesized that in order to maintain expression without a 3'UTR being present in the *pbcr* genomic locus, parasites employed alternative 3'UTRs to stabilize *pbcr* mRNAs and permit protein production. To identify any such sequences, 3' rapid amplification of cDNA ends (3'RACE; Scotto-Lavino et al., 2006) was performed on the transmitted, recloned parasites from both deleter backgrounds (T9-rc and U4-rc). These 3'RACE reactions produced a single dominant band of just over 0.5 kb in T9-rc, and several bands of different length in clone U4-rc (ca. 0.25, 0.3-0.4, 0.55, 0.7 and 2.2 kb) (Fig. 3A). These products included the last 0.24 kb of *pbcr* plus the alternative 3'UTRs. Most of the observed sizes were therefore consistent with the only previously characterized *Plasmodium* 3'UTRs (~0.45 kb for *Plasmodium gallinaceum* *pgs28* (Golightly et al., 2000) and 0.16-0.25 kb for *pfcpna* (Wong et al., 2011)). The 3'RACE products were gel-extracted and ligated into a pGEM-T Easy vector (Promega, USA), and five and 14 *pbcr*-specific independent plasmids were sequenced from the T9-rc and U4-rc reactions, respectively. Fig. 3B illustrates the identified sequences.

In 3' RACE products from the U4-rc clone, we observed three different types of mRNA (Fig. 3B). First, in five pGEM-T Easy clones a poly(A) tail was directly attached to the *pbcr* fully spliced coding sequence upstream of the stop codon. Two different attachment sites were observed (-10 and -77 nucleotides from the stop codon) with the resulting PbCRT protein lacking the last four or 26 C-terminal amino acids, respectively. Second, in seven pGEM-T Easy clones a poly(A) tail was attached to the plasmid backbone that remained in the *pbcr*-no3'UTR locus, with attachment sites observed at either +112, +214 or +448 nucleotides from the stop codon. In this second set of sequences, therefore, the entire *pbcr* coding sequence was retained, followed by the *NotI* restriction site used for cloning the p*Pbcr*-cKO plasmid, the single *FRT* site remaining after site-specific recombination, and 62, 164 or 398 nucleotides of plasmid backbone, respectively. These sequences were followed by the poly(A) tail. Third, in two pGEM-T Easy clones a poly(A) tail was attached to an unspliced *pbcr* sequence upstream of its stop codon. This resulted in PbCRT being prematurely terminated with the last 58 C-terminal amino acids being replaced by six different amino acids. These RACE-PCR products were of mRNA origin, and not due to genomic DNA contamination, as no product was obtained in a control RACE-reaction that did not include reverse transcriptase.

For T9-rc, four of the five bacterial clones contained the same sequence (sequence A), with the fifth bacterial clone containing a very similar sequence (sequence B). In these sequences the 3' end of *pbcr* was followed by an inverted *trap* 3'UTR followed by a poly(A) tail. Importantly, at the genomic level the T9-rc clone had the expected arrangement of the *pbcr*-no3'UTR locus (Fig. 1A), as assessed by diagnostic PCR and sequencing. This suggests that the *pbcr* mRNAs acquired the inverted *trap* 3'UTR sequence during the process of

transcription. Of note, the *P. berghei trap* genomic locus is located on chromosome 13, as opposed to chromosome 12 for *pbcr*. Interestingly, the *trap* gene is positioned in a tail-to-tail arrangement with PBANKA_134970 (non-annotated), with only 0.6 kb separating the two stop codons. It is therefore quite likely that the *trap* 3'UTR is bidirectional and the inverted sequence might therefore provide a functional 3'UTR when joined onto the 3' end of the *pbcr* coding sequence. Interestingly, this inverted *trap* 3'UTR was connected imperfectly to the *pbcr* coding sequence, resulting in a modified PbCRT C-terminus. In sequence A, the last 15 PbCRT amino acids (NDSEAELTSIETSRA*; where * denotes the C-terminal end of the sequence) were replaced with YDHDYAKRAINPH*, while in sequence B the last three PbCRT amino acids (SRA*) were replaced with AMTMITPSAQLTLTKGNKSWSLGGPSIIFVS*.

It is worth pointing out that the anti-PfCRT monoclonal antibodies used herein are directed to a C-terminal amino acid stretch (residues 401-419; (Fidock et al., 2000)) that was lost from sequence A of T9-rc and from some of the U4-rc alternate mRNA species (Fig. 3B). Our western blots of clones T9-rc and U4-rc (Fig. 2C) are therefore qualitative and cannot provide quantitative assessment of the level to which PbCRT protein expression was preserved. It is clear, however, that at least some of the alternate 3'UTRs identified retain function, both in T9-rc and U4-rc parasites, such that PbCRT protein can still be produced. Of note, neither T9-rc nor U4-rc displayed any reduction in growth compared with the parental lines (Fig. 2A). Given that in *P. falciparum* a reduction of PfCRT levels by 30-40% (achieved through truncation of the 3'UTR) resulted in slower growth (Waller et al., 2003), the lack of a growth phenotype in our *pbcr*-no3'UTR clones is further evidence for the ability of the aberrant 3'UTRs to maintain *pbcr* expression.

Of note, we have recently learned that our p*Pbcr*-cKO plasmid design differed subtly from plasmids described in the earlier Flp/*FRT* reports (Combe et al., 2009; Lacroix et al., 2011) in that our plasmid was missing the first 12 nucleotides of the *trap* 3'UTR. This did not appear to affect expression of *pbcr* prior to excision, nor did it impact excision of the *FRT*ed sequence (see Fig. 1), which was found to be highly efficient at the genomic DNA level by quantitative PCR, and was verified by sequence analysis. This sequence difference therefore seems to be an unlikely explanation for the aberrant *pbcr* mRNAs observed in the post-excision parasites. The lack of this short sequence stretch constitutes the only difference between our construct and plasmids used to successfully silence other genes (Combe et al., 2009; Lacroix et al., 2011), and as such provides a possible explanation for the production of aberrant *pbcr* mRNAs observed in our post-excision parasites.

In summary, *pbcr* expression was maintained following removal of its 3'UTR in independent clones derived from two different deleter strains. Several alternative 3'UTR sequences were identified (Fig. 3B), underscoring the astounding plasticity of this parasite at the nucleic acid level. Notably, following mosquito bite or direct inoculation with post-excision blood-stage parasites, mice became blood smear-positive with clones T9 and U4 with no delay compared with the parental deleter lines TRAP/FlpL and UIS4/Flp, respectively. This suggests that these modified *pbcr* mRNAs are readily generated. We cannot be certain that all of these mRNAs result in the production of functional PbCRT protein. However, given the continued expression of PbCRT in both T9-rc and U4-rc, we are confident that at least some of these alternate 3'UTRs are able to sufficiently stabilize *pbcr* mRNAs for successful translation into protein. These sequences may therefore provide insights into the necessary characteristics of endogenous *Plasmodium* 3'UTRs, which remain largely undefined (Horrocks et al., 2009). These findings also call for careful molecular analysis of cKO events based on 3'UTR excision, as *Plasmodium* may adopt alternative elements to overcome gene silencing. In addition, our observations demonstrate that the parasite is under enormous selective pressure to maintain *pbcr* expression,

consistent with this protein product having an important or essential function in the stages following the 3'UTR excision process.

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Highlights

- We sought to conditionally silence the chloroquine resistance transporter to test its role across the *Plasmodium* lifecycle.
- This used the Flp/FRT system to excise the 3'UTR during mosquito passage, leading to unstable, non-functional transcripts.
- Despite successful excision, *P. berghei* progressed normally through the lifecycle by attaching alternative 3'UTR elements.
- This study highlights the remarkable ability of *Plasmodium* at the genomic level, to overcome disruption of essential genes.

Fig. 1.

Generation of *Plasmodium berghei* chloroquine resistance transporter (*pbcrt*) conditional knockout (cKO) parasites via 3' untranslated region (UTR) excision mediated by Flp/*FRT* site-specific recombination. (A) Schematic of the double crossover event between the linearized transfection plasmid and the endogenous *pbcrt* locus, resulting in the pre-excision *pbcrt-trap* 3'UTR locus and the post-excision *pbcrt-no3'UTR* locus. The structure of the 13-exon *pbcrt* gene is shown stylistically, with introns in grey and exons in white. The thick black line denotes the plasmid backbone. Flp, Flp recombinase; *FRT*, Flp recognition target; *trap*, thrombospondin related adhesive protein; *hdhfr*, human dihydrofolate reductase selectable marker. H, *HindIII*; N, *NotI*; S, *SphI*. (B) PCR analysis of the *pbcrt* locus pre- and post-excision. The non-excised locus was detected using primers p5 + p6 (that yield a 1.5 kb band). The excised locus, post mosquito passage, was detected using primers p5 + p7 (yielding a 0.86 kb product), and primers p1 + p7 (0.73 kb). These primer pairs can also amplify the non-excised locus, yielding 3.1 and 3.0 kb products, respectively, in the pre-excision parasites (the presence of the 0.6 kb band in those lanes and in the post-excision bulk blood stages was determined by sequencing to be off-target amplification of the mouse integrin α -8 gene). Absence of the wild-type *pbcrt* locus was confirmed in all recombinant parasites using primers p1 + p4 (that yield a 1.2 kb product in wild-type parasites; data not shown). These primers yielded a large (>>3 kb) product spanning the plasmid backbone in the *pbcrt-no3'UTR* locus in post-excision blood stage parasites (this product would theoretically also be present in sporozoites with an excised locus, but it is difficult to amplify a product of this size from the low yield of parasite genomic DNA that can be obtained from infected mosquitoes). Excision of the *trap* 3'UTR was not observed in blood stage parasites prior to mosquito transmission, but was evident in midgut and salivary gland sporozoites. Following retransmission to naïve mice, most but not all blood stage parasites had excised the *FRT*ed *trap* 3'UTR locus. The re-cloned post-excision parasites T9-rc and U4-rc harbor the excised locus (*pbcrt-no3'UTR*) exclusively.

Fig. 2.

Post-excision parasites harboring *pbcr*-no3'UTR (*Plasmodium berghei* chloroquine resistance transporter - no3' untranslated region) show no marked loss of fitness and express *pbcr* transcripts and protein product. (A) Growth kinetics of post-excision (T9-rc and U4-rc) and parental strains (TRAP/FlpL and UIS4/Flp) propagated in mice. Parasitemias are shown as means $\pm$ S.E.M. calculated from groups of 10 mice per line. (B) Reverse transcriptase (RT)-PCR analysis of pre-excision T9 blood stage parasites and the post-excision clone T9-rc. *pbcr* and *P. berghei* apical membrane antigen 1 (*pbama1*, PlasmoDB ID: PBANKA_091500) transcripts were detected from parasite cDNA using primers p12 + p13 (yielding a 0.74 kb band) and p14 + p15 (yielding a 1.0 kb band), respectively. + and - RT denote reactions with and without reverse transcriptase. (C) PbCRT expression was assayed by western blot hybridization with saponin-lysed protein extracts from the post-excision clones (T9-rc and U4-rc) and the parental deleter strains. Membranes were stained for PbCRT using a mouse monoclonal anti-PfCRT hybridoma supernatant (monoclonal antibody (mAb) CU1711.626 generated against the synthetic PfCRT peptide (KKMRNEENEDSEGELTNVDC), diluted 1:100; Covance, USA) followed by incubation with a horseradish peroxidase-conjugated sheep anti-mouse IgG (NXA931 diluted 1:10,000; GE Healthcare, USA), and then detected using a chemiluminescent substrate (Pico West; Thermoscientific, USA). Membranes were then re-probed with rat monoclonal anti-*P. berghei* AMA1 antibody (mAb 28G2 diluted 1:100; (Kocken et al., 1998)) followed by incubation with a horseradish peroxidase-conjugated goat anti-rat IgG (NA935V diluted 1:10,000; GE Healthcare) and detection by chemiluminescence (ECL Plus; GE Healthcare). Each lane contains protein extract from a separate mouse infected with the indicated parasite line. Differences in band intensity are likely to reflect stage-dependent variation in levels of PbCRT and AMA1 expression (from samples collected at different times) as well as variable protein loading.

Fig. 3.

Post-excision re-cloned parasites with the *pbcr1*-no3'UTR locus (*Plasmodium berghei* chloroquine resistance transporter - no3' untranslated region) express *pbcr1* mRNA with a variety of alternative 3' regulatory sequences. (A) 3' rapid amplification of cDNA ends (RACE) products for T9-rc and U4-rc. mRNA was reverse transcribed using primer Q_T (Scotto-Lavino et al., 2006). In the first round of PCR amplification, *pbcr1* 3' cDNA sequences (comprising the last 0.3 kb of coding sequence extending into the poly(A) tail) were amplified using primers p5 and Q_O. In the nested PCR (shown here), *pbcr1* 3' cDNA ends (comprising the last 0.24 kb of coding sequence plus the 3' regulatory sequence) were amplified using primers p1 and Q_I. A single dominant band was observed for clone T9-rc, and several bands for clone U4-rc. PCR products were gel-extracted, ligated into a pGEM-T Easy plasmid and sequenced with vector-specific primers SP6 and M13F (Table 1). (B) Schematic representation of alternative 3'UTRs observed in *pbcr1* mRNAs from post-excision *pbcr1* conditional knockout (cKO) parasites. The exon-intron structure of the 13-exon *pbcr1* coding sequence is shown stylistically as grey (intron) and white (exon) boxes. All but one of the observed patterns resulted in the production of PbCRT protein with a modified C-terminus. PCR and sequencing analyses indicated that these alternative 3' regulatory elements were added during transcript processing, as the post-excision *pbcr1*-no3'UTR genomic locus lacked a proximal 3'UTR.

Table 1

List of primers used in this study.

Name	Nucleotide Sequence	Description
p1	5′ -cc <u>G</u> CATGCTACACCATTGTTAGTTGTATACAAGG	Forward primer of <i>Pbcr</i> 3′ coding sequence; <i>Sph</i> I site underlined
p2	5′ -tt <u>GCGGCCG</u> CTTATGCCCTTGATGTTTCTATAGAAG	Reverse primer of <i>Pbcr</i> 3′ coding sequence; NotI site underlined
p3	5′ -cc <u>AAGCTT</u> TTGATACAACATTTTATTTCTTAAATGATTTTGT	Forward primer of <i>Pbcr</i> 3′ UTR; <i>Hind</i> III site underlined
p4	5′ -cc <u>G</u> CATGCTCTCTATACATAGGCAAATAAGG	Reverse primer of <i>Pbcr</i> 3′ UTR; <i>Sph</i> I site underlined
p5	5′ -CATATGTGATAATTTACTTGCTTGC	Forward primer of <i>Pbcr</i> upstream coding sequence
p6	5′ -CTGGTGCTTTGAGGGGTGAGC	Reverse primer from <i>hdhfr</i> selectable marker cassette
p7	5′ -CAGGAAACAGCTATGAC	Reverse primer specific to the plasmid backbone
p8	5′ -GTTGGTTCGCTAAATCGCATC	Forward primer for <i>hdhfr</i> qPCR
p9	5′ -CTGTTTACCTTCTACTGAAGAGC	Reverse primer for <i>hdhfr</i> qPCR
p10	5′ -TGCAGCAGATAATCAAACCTC	Forward primer for <i>hsp70</i> qPCR
p11	5′ -ACTTCAATTTGTGGAACACC	Reverse primer for <i>hsp70</i> qPCR
p12	5′ -CCTTATCTCATTATTAGATGCTTCTAC	Forward primer for <i>Pbcr</i> RT-PCR
p13	5′ -CCAATATTCTTGGTTTTCTTACAGC	Reverse primer for <i>Pbcr</i> RT-PCR
p14	5′ -TATGGGTCCAAGATATTGTAGTAATAA	Forward primer for <i>Plasmodium berghei</i> <i>ama1</i> RT-PCR
p15	5′ -GAATTAGCTTTACCATAAATATCTGC	Reverse primer for <i>P. berghei</i> <i>ama1</i> RT-PCR
Q _T	5′ -CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC(T) ₁₇	Primer to generate cDNA for 3′ RACE
Q _O	5′ -CCAGTGAGCAGAGTGACG	Outer primer for 3′ RACE
Q _I	5′ -GAGGACTCGAGCTCAAGC	Inner primer for 3′ RACE
SP6	5′ -ATTAGGTGACACTATAG	pGEM vector-specific primer to sequence 3′ RACE products
M13F	5′ -GTAAACGACGGCCAGT	pGEM vector-specific primer to sequence 3′ RACE products

ama1, apical membrane antigen 1; *hdhfr* human dihydrofolate reductase selectable marker; *hsp70*, heat shock protein 70; *Pbcr*, *Plasmodium berghei* chloroquine resistance transporter; qPCR, quantitative PCR; RACE, random amplification of cDNA ends; RT-PCR, reverse transcription PCR; UTR, untranslated region.