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Alpha-tocopherol transfer protein disruption confers resistance to malarial infection in mice

Maria S Herbas[1,5], Yoshiko Y Ueta[1], Chie Ichikawa[1], Mayumi Chiba[1], Kana Ishibashi[1], Mototada Shichiri[2], Shinya Fukumoto[1], Naoaki Yokoyama[1], Motohiro Takeya[3], Xuenan Xuan[1], Hiroyuki Arai[4] and Hiroshi Suzuki*[1,5]

Abstract

Background: Various factors impact the severity of malaria, including the nutritional status of the host. Vitamin E, an intra and extracellular anti-oxidant, is one such nutrient whose absence was shown previously to negatively affect *Plasmodium* development. However, mechanisms of this *Plasmodium* inhibition, in addition to means by which to exploit this finding as a therapeutic strategy, remain unclear.

Methods: α-TTP knockout mice were infected with *Plasmodium berghei* NK65 or *Plasmodium yoelii* XL-17, parasitaemia, survival rate were monitored. In one part of the experiments mice were fed with a supplemented diet of vitamin E and then infected. In addition, parasite DNA damage was monitored by means of comet assay and 8-OHdG test. Moreover, infected mice were treated with chloroquine and parasitaemia and survival rate were monitored.

Results: Inhibition of α-tocopherol transfer protein (α-TTP), a determinant of vitamin E concentration in circulation, confers resistance to malarial infection as a result of oxidative damage to the parasites. Furthermore, in combination with the anti-malarial drug chloroquine results were even more dramatic.

Conclusion: Considering that these knockout mice lack observable negative impacts typical of vitamin E deficiency, these results suggest that inhibition of α-TTP activity in the liver may be a useful strategy in the prevention and treatment of malaria infection. Moreover, a combined strategy of α-TTP inhibition and chloroquine treatment might be effective against drug resistant parasites.

Background

Despite recent advances in understanding malaria and *Plasmodium*, the parasite responsible for the disease, 500 million cases of clinical malarial in over 100 countries still occur. This disease poses a public health problem for 3.3 billion people, a number representing a staggering 50% of the world's population. Furthermore, the global death figure for malaria reaches more than 1 million each year [1]. A number of factors affect the severity of malaria, including the size of the sporozoite infective dose, host nutritional status, acquired immunity level, host genetic factors, parasite features and even certain associated socioeconomic factors [2-7]. Although micronutrient malnutrition is usually highly prevalent in areas in which malaria is endemic, the contribution of these micronutrient deficiencies to malarial symptoms is often overlooked.

Vitamin E is a powerful anti-oxidant that acts mainly in the lipid phase of cells and has a primary role in preventing the oxidation of polyunsaturated fatty acids [8]. While vitamin E deficiency seems to have both protective and adverse effects in malarial infection, the involvement of vitamin E in the genesis of malarial illness is still controversial [9]. The clinical observations that feeding famine victims with grain exacerbated the effects of cerebral malaria were attributed to the vitamin E content of the grain that subsequently influenced severity of malaria symptoms [10]. In addition, according to the results of animal studies, dietary vitamin E deficiency is thought to protect against malarial infection, presumably because the absence of this anti-oxidant leads to an increase in oxygen radicals production derived from the immune response of the host against the infection, consequently making an inhospitable environment for the parasite

* Correspondence: hisuzuki@obihiro.ac.jp
[1] Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13, Inada, Obihiro, 080-8555 Japan
Full list of author information is available at the end of the article

[11,12]. However, even if it were shown to be possible to utilize vitamin E deficiency for the prevention or treatment of malaria, it would be quite difficult to actually lower vitamin E in circulation via nutritional manipulation because the majority of daily foods in a normal diet contain significant amounts of vitamin E [8].

Vitamin E is transported in plasma lipoproteins and, unlike other fat-soluble vitamins, has no specific plasma carrier protein, however, alpha-tocopherol transfer protein (α-TTP), a liver cytosolic protein, acts as an important regulator of vitamin E concentration in circulation [13,14]. It does this through binding specifically α-tocopherol amongst the other tocopherols, including β and γ-tocopherol, in the liver. Targeted disruption of the *α-TTP* gene revealed that α-tocopherol concentration in circulation was regulated by *α-TTP* [13,15]; heterozygous mutant mice contained plasma concentrations of α-tocopherol half that found in wild type mice while homozygous mutants were shown to have undetectable levels of α-tocopherol in circulation [14]. Actual mechanism is not known. However, it is postulated that chylomicrons remnants with extra amount of α-tocopherol leaked in to the circulation.

The ability to manipulate α-tocopherol levels, and consequently vitamin E levels, via *α-TTP* gene inhibition inspired us to revisit the impact of serum vitamin E levels on the severity of malarial infection using an established rodent malarial model. Thus, *α-TTP* inhibition was examined as a potential application for the prevention or treatment of malarial infection. The results indicated that *α-TTP* inhibition led to parasite DNA damage sufficient to inhibit proliferation. Moreover, a combined therapy with chloroquine (CQ) seems to be useful as a new strategy for the treatment of malaria.

Methods

Animals and malaria infection

Since C57BL/6J mice are frequently used as an experimental model in biomedical sciences, α-TTP knockout mice were generated with a C57BL/6J background [14]. These knockout mice were bred in our own colony. BALB/c mice were known to be murine malaria resistant strain. Adult α-TTP knockout mice with a C57BL/6J genetic background, C57BL/6J and BALB/c mice were infected with 4×10^5 *Plasmodium berghei* NK65 or 4×10^4 *Plasmodium yoelii* 17XL infected red blood cells (IRBCs) by intraperitoneal injection, and their survival was monitored. Six animals were used in each experimental group. Mice were fed with a commercial diet (CE-2, containing 45 mg/kg of D-α-tocopherol, CLEA Japan, Tokyo, Japan) or α-tocopherol supplementation (CE-2 with supplementary D-α-tocopherol, 600 mg/kg, CLEA Japan, Tokyo, Japan). Furthermore, to determine the effect of vitamin E deficiency on the virulence of *P. berghei* NK65, α-TTP knockout or C57BL/6J mice were inoculated with *P. berghei* NK65 (4×10^5 IRBCs) recovered from α-TTP knockout or wild type mice at day 9 post-infection, and their survival was monitored. In a part of the experiments, mice infected with *P. berghei* NK65 were administered 0-7.5 mg/kg of CQ (C6628, Sigma-Aldrich, St. Louis, USA) on day 0, 1 and 2 after infection, and their survival was monitored. All experiments described in the present study were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals of the Obihiro University of Agriculture and Veterinary Medicine, Japan.

Haematological analysis

Haematological parameters, such as the number of red blood cells (RBCs), white blood cells (WBCs), haemoglobin concentration, and haematocrit were determined with an autohaematology analyzer (Celltac α, MEK-6358, Nihon Kohden, Tokyo, Japan) during the course of infection. Six mice were used for each experimental group. For the reticulocyte count, 2 μl of whole blood taken from the tail vein were mixed with 2 μl of Brilliant Crystal Blue (Wako, Tokyo, Japan). Then thin smears of sample were prepared, dried at room temperature and stained with Giemsa (Merck, Darmstadt, Germany). The numbers of infected and uninfected reticulocytes, as well as mature RBCs, were determined under light microscopy.

The comet assay

DNA damage of the parasites infecting the mice was assessed by single-cell gel electrophoresis (comet assay) [16]. Evaluation of the shape of the DNA "comet" tail and migration pattern gives an assessment of DNA damage. Whole blood was obtained by cardiac puncture, washed with cold phosphate buffered saline (PBS), and then centrifuged twice at 5,000 rpm for 5 min at 4°C. After removing the buffy coat, RBCs were washed twice in PBS. Cell mixture was suspended in Comet LMAgarose (1% low-temperature-melting agarose, Trevigen, Gaithersburg, MD, USA) at a ratio of 1:10 (v/v), 25 μl of the cell suspension was immediately placed on a CometSlideTM (Trevigen), slides were placed flat in a refrigerator at 4°C for 10 min, and then submerged in 23 ml of lysis solution (Trevigen) at 4°C for 60 min. Subsequently, slides were then maintained in an alkaline solution (>pH13) for 60 min at room temperature in the dark and washed two times in 1× TBE buffer (Tris-borate EDTA) for two times (5 min. each). Finally, slides were subjected to electrophoresis in 1× TBE buffer at 25 V for 10 min, stained with SYBR Green (Trevigen), and analysed under microscopy (IX-70, Olympus Co., Tokyo, Japan). Denatured cleaved DNA fragments migrated out of the cell under the stimulus of an electric potential, whereas the undamaged supercoiled DNA remained within the confines of the cell

membrane when a current was applied. Parasites from α-TTP knockout with 18-29% of parasitaemia were recovered on day 21 after the infection. Parasites from C57BL/6J with 40-49% of parasitaemia were collected on day 12 after the infection. Four mice were used each experimental group.

Detection of biomarker for oxidative stress with anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody

Collected blood from infected animals (n = 4) was mixed with an equivalent volume of PBS and then was centrifuged at 5,000 rpm for 5 min at 4°C, then the supernatant was removed (this step was repeated 3 times). The percentages of parasitaemia in α-TTP knockout and C57BL/6J mice were 27% and 35%, respectively. The pellet was resuspended with 0.1 ml of PBS and 0.1 ml of 3% foetal calf serum. The suspension was fixed with methanol for 10 min. Anti-8OHdG (2 ng/ml), a biomarker for the oxidative damage of DNA [17], monoclonal antibody (N45.1; MOG-20P; Japan Institute for the Control of Aging, Nikken SEIL Co. Ltd., Shizuoka, Japan) was labelled with Biotin-XX Mouse IgG1 according to the manufacturer's instructions (Z25052: ZenonTM Biotin-XX mouse IgG1, Molecular Probes, Eugene, USA), then this complex was incubated with the samples for 45 min at 37°C. The sample was washed twice with PBS for 5 min, and was incubated with streptavidine-Alexa Fluor 488 conjugate (Molecular probes) for 45 min at 37°C. The incubated sample was washed twice with PBS for 5 min. Parasite DNA was stained with propidium iodide (P1304 MP; Molecular Probes) containing RNase A (10109142001; Roche Applied Science, Mannheim, Germany) for 10 min at 37°C. After washing with PBS, the sample was treated with 1% n-propyl gallate (102747; MP Biomedicals, Irvine, CA USA), an anti-oxidant, and observed with a confocal laser microscope (DMRB/E, TCS NT; Leica Microsystems, Wetzlar, Hessen, Germany).

Quantification of anti-oxidative stress enzymes of P. berghei NK65

The mRNA expression of anti-oxidative stress enzymes of *P. berghei* NK65, such as glutaredoxin (Grx), γ-glutamil transferase (γ-GCS), 2-Cys peroxiredoxin (2-Cys Prx) and thioredoxin reductase (TrxR), was monitored by a real time quantitative PCR carried out with specific double labelled probes in the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, CA, USA). RBCs from infected and uninfected mice were separated from whole blood cells by using a gradient reagent (Histopaque-1077, Sigma, MO, USA). The sample was washed with cold PBS followed by centrifugation at 5,000 rpm for 10 min at 4°C in order to remove the residual buffy coat. This step was repeated twice. Then, the separated RBCs were treated with 0.15% saponin (Sigma-Aldrich, St. Louis, USA) and then were centrifuged and washed with cold PBS twice. The total RNA of parasites was extracted from the pellet by using TRI reagent (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions, and successively treated with Turbo DNA-freeTM reagent (Ambion, Texas, USA). The total RNA concentration was adjusted to 50 ng/μl. The mRNA amplification reaction (20 μl) consisted of 10 μl of 2× master mix without UNG, 0.5 μl of 40× multiscribe and RNase inhibitor mix, 1.8 μl of 10 μM of each forward and reverse primers, 0.8 μl of 5 μM TaqMan Probe, 4 μl of total RNA template (50 ng/μl) and 1.1 μl of RNase free double distilled water. The real time quantitative PCR first stage conditions were: 48°C for 30 min and the second stage of 45 reaction cycles with the following conditions: 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min. The primers and probes used are shown in Table 1. Standard amplification curves were obtained by serial dilution of the total RNA of parasites. *Plasmodium berghei* 18SrRNA was used as an internal control. Six mice were used for each experimental group.

Statistical analysis

Statistical analysis was performed using one-way variance analysis (S-plus6 software for windows) (Insightful corporation, Seattle Washington USA). Data are expressed as means of the standard error of the mean (SEM). P values less than 0.05 were considered to be significant. Survival rate % analysis was performed using the Kaplan-Meier method.

Results

Resistance of α-TTP knockout mice to malaria infection

When α-TTP knockout mice were infected with a lethal dose of *P. berghei* NK65 IRBCs, their survival was significantly extended compared to wild type (C57BL/6J) mice ($p < 0.01$) (Figure 1a). Furthermore, knockout mice harboured low parasite loads during the initial peak of parasitaemia, around day 10 post infection, when most wild type mice were dying (around day 12 post infection) (Figure 1b). Survival was longer and parasitaemia was lower in the α-TTP knockout than *P. berghei* NK65 resistant BALB/c and wild type mice. Lastly, inhibition of α-TTP did not trigger the development of anaemia during the acute phase of the infection; the total number of RBCs in knockout mice was $764 \pm 47 \times 10^4$/ul, comparable to wild type mice ($871 \pm 62 \times 10^4$/ul). The effect of the α-TTP gene disruption was much more remarkable for a *P. yoelii* 17XL infection that killed nearly all wild type mice by day 8; all knockout mice survived (Figure 1c). Furthermore, while some α-TTP knockout mice exhibited a trace level of parasitaemia at an early phase of infection, the IRBCs subsequently disappeared from circulation (Figure 1d). Taken together, it appears that knockout of *α-TTP* leads

Table 1: Primer and Probe sequences used in real--time quantitative PCR

Gene	Primer/probe
18s rRNA	5'-CGATGTGTGTCTAACACAAGGAAGT-3'F
	5'-CATAGGCTTTAACACCTAAGCACAG-3'R
	5'-FAM-TATGTAAAACGAGTGTTAAAT-MGB-3'
2-Cys Prx	5'-AAACACCATTGTCACAAGGAGGTA-3'F
	5'-ACAAACGCTCTTAATGCTACATTTC-3'R
	5'-FAM-AAGCATACTTTGATATCCG-MGB-3'
g-GCS	5'-AATGAGTATGTGCTGCCAACAAAT-3'F
	5'-AAGAATCCACCTAAATATGGTGTACATG-3'R
	5'-FAM-AGCTAGCTGTAATTGC-MGB-3'
Grx	5'-GGGAAAAGTTCCGTACCAAGAAT-3'F
	5'-TTCCAATGTCTGGAGTCTCTCTGA-3'R
	5'-FAM-ACCAGTCATCACATCCGCCGACATT-MGB-3'
TrxR	5'-AGTCACTCAAGGAATGGGATTGG-3'F
	5'-TGCATAAGACAAACCCGATGATAA-3'
	5'-FAM-TTCATCCAACAGATGCAG-MGB-3'

to inhibition of parasite development and subsequent longevity of the animals in the face of infection.

The spleen plays an important role during malarial infection removing uninfected and infected red blood cells from circulation. Histological analysis of spleens revealed the presence of haemozoin, a toxic haem product derived from the haemoglobin digestion thought to be an indication of macrophage phagocytic activity and parasite maturation [18], in the red pulp was visible by day 6 that increased in intensity by day 9 in wild type animals (Figure 1e). In addition, during the acute phase of the infection an increase in mature RBCs could be found indicating erythropoietic activity might be diminished in the wild type mice, a condition that could lead to anaemia. In contrast to wild type animals, α-TTP knockout mice displayed very little pigmentation or increase in mature RBCs even by day 9 indicative of delayed parasite maturation in these mice. In addition, mature RBCs and erythroblasts were intermixed in the red pulp of the spleen in α-TTP knockout mice indicating that red blood cell production is not affected in the infected mice early after infection. Moreover, the mRNA expression of erythropoietin receptor (EPOR) in bone marrow was significantly decreased in both genotypes on day three post infection, however the expression of EPOR was significantly increased in liver and spleen suggesting such organs compensate bone marrow function in order to avoid anaemia during the acute phase of the infection (manuscript under submission). These results indicate that α-TTP knockout animals are displaying no signs of infection even at a time when most wild type animals are dying.

Plasma concentrations of vitamin E in the knockout and wild type mice fed to a normal diet were 0.02 ± 0.01 μM and 0.98 ± 0.12 μM, meanwhile, concentrations of vitamin E with the supplemented diet was 1.18 ± 0.08 μM and 3.3 ± 0.2 μM, respectively. Then, to analyse whether restoration of circulating vitamin E concentration in α-TTP knockout mice could lead to malaria susceptibility, mice were fed a diet supplemented with α-tocopherol (600 mg/kg diet) ten days before infection with *P. berghei* NK65. Survival curves and parasitaemia kinetics of knockout mice fed an excess amount of α-tocopherol-supplemented diet, were similar to wild type (Figures 2a-b). These results demonstrate that α-TTP knockout mice acquired resistance to malaria infection by vitamin E deficiency, not by *α-TTP* gene disruption itself.

DNA damage of Plasmodium in α-TTP knockout mice

Since decreased vitamin E concentrations likely lead to an environment of high oxidative stress, DNA damage in these parasites was assessed. A comet assay evaluates the shape of the DNA "comet" tail and migration pattern as an indication of DNA damage [16] and revealed that the parasite-infected RBCs in α-TTP knockout mice had severe DNA damage (Figure 3a). This was in contrast to infected RBCs from wild type mice where parasites displayed no such comet tails. Immunofluorescence staining of anti-8 hydroxy-2-deoxyguanosine (8-OHdG), a biomarker for oxidative DNA damage [17], confirmed oxidative DNA damage to parasites recovered from α-TTP knockout mice (Figure 3b). Taken together it appears that loss of α-TTP leads to DNA damage of hosted *Plasmodium* parasites, presumably due to consequent decreased vitamin E concentration and its associated free radical scavenging activity.

To elucidate whether the virulence of parasites in α-TTP knockout mice might be affected during infection due to parasite DNA damage, mice were inoculated with *P. berghei* NK65 that had been recovered from previously infected α-TTP knockout or wild type mice 9 days post infection. Tellingly, both the α-TTP knockout and wild type mice infected with parasites recovered from the α-TTP knockout mice survived significantly longer than the mice infected with parasites recovered from wild type mice (Figure 3c). Surprisingly, despite increased longevity, parasitaemia kinetics in wild type mice and knockout mice were similar irrespective of the source of the parasites (Figure 3d). These results suggest that the virulence of the parasites existing in α-TTP knockout mice had decreased during their time within that host.

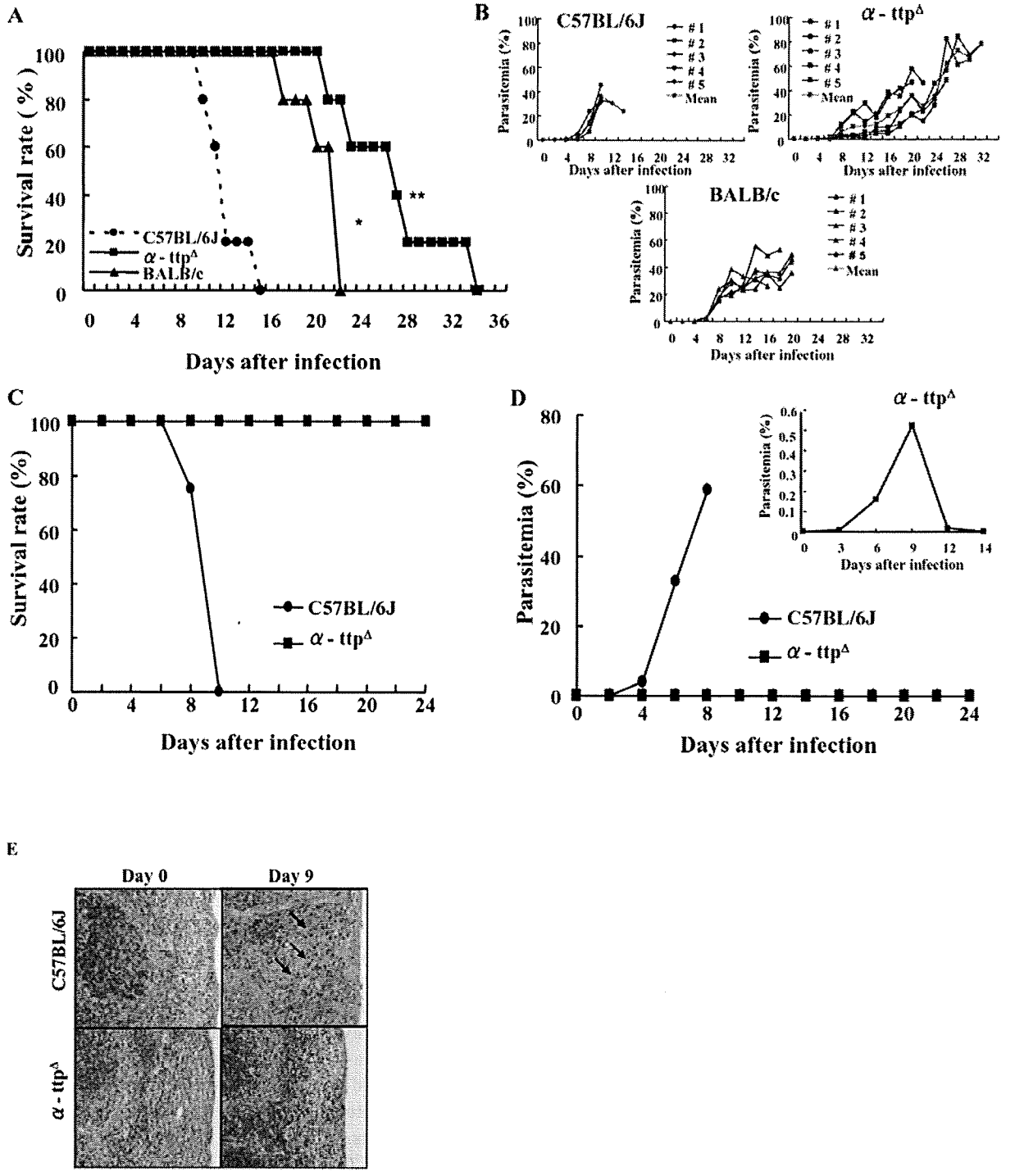

Figure 1 Parasite proliferation inhibition in α-TTP knockout mice. (A) Survival rates of α-TTP knockout mice (α-ttpΔ) infected with *P. berghei* NK65 were significantly longer than that of BALB/c and C57BL/6J mice ($p < 0.01$). (B) Percentages of parasitaemia for individual animals of each genotype. (C) Percentage of parasitaemia of α-TTP knockout mice infected with *P. yoelii*. (D) All α-TTP knockout mice infected with *P. yoelli* 17XL survived with lower parasitaemia. The inset graph refers to a different scale of parasitaemia. (E) Malaria pigment (arrows) seen in the spleen of C57BL/6J mice but not in α-TTP knockout mice on day 9 after infection with *P. berghei* NK65 ($n = 6$). *: $p < 0.05$ C57BL/6J vs BALB/c; **: $p < 0.01$ C57BL/6J vs α-ttpΔ in panel A.

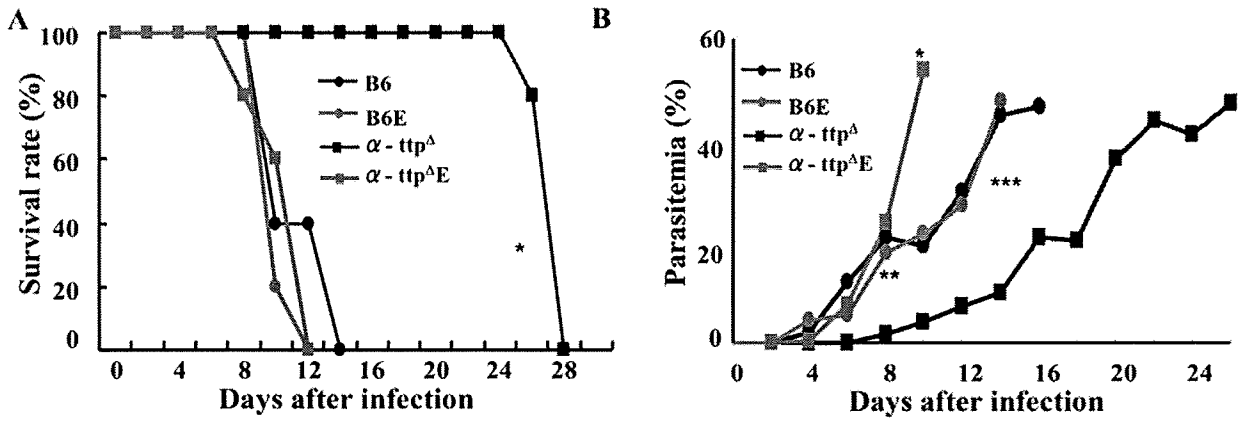

Figure 2 Vitamin E supplementation reversed α-TTP knockout conferred resistance. (A) Survival rate of α-TTP knockout mice fed with vitamin E supplementary diet (α-ttp$^{\Delta}$E) and infected with *P. berghei* NK65 was similar to the C57BL/6J mice fed to a normal diet (B6) or C57BL/6J mice fed to a supplemented diet of vitamin E (B6E). (B) Percentages of parasitaemia of α-TTP knockout mice fed with vitamin E supplementary diet was similar to the C57BL/6J mice. (n = 6) *: $p < 0.05$ B6, B6E, α-ttp$^{\Delta}$E vs α-ttp$^{\Delta}$ in panel A. In panel B *: $p < 0.01$ α-ttp$^{\Delta}$ vs α-ttp$^{\Delta}$E; ** $p < 0.01$ α-ttp$^{\Delta}$ vs B6E on day 10 post infection; ***: $p < 0.01$ α-ttp$^{\Delta}$ vs B6, B6E and α-ttp$^{\Delta}$E on day 14 post infection.

Expression of anti-oxidative enzymes in infected parasites

After infection with *P. berghei* NK65, the mRNA expression of anti-oxidative stress enzymes, such as glutaredoxin (Grx), γ-glutamil cysteine synthetase (γ-GCS), 2-Cys peroxiredoxin (2-Cys Prx) and thioredoxin reductase (TrxR), of the parasites was examined using real time quantitative PCR. Two key anti-oxidant genes that have been used frequently to monitor the status of the anti-oxidant machinery of parasites, such as *γ-GCS* and *Grx*, showed higher levels of expression at initial points in the knockout mice compared to the wild type mice (Figures 4a-b). Parasites from knockout mice showed steady levels of *TrxR* expression until day 12 post-infection when it dropped (Figure 4c). These levels were comparable to those of parasites from wild type hosts. However, *TrxR* expression dropped dramatically at day 9 before recovering at day 12 in the wild type mice. Despite the similar levels of expression in *TrxR* between knockout and wild type mice, it appears that parasites have activated anti-oxidant systems at the earliest time points of infection in the knockout mice. Increased transcription of anti-oxidant genes in infected α-TTP knockout mice was in contrast to infected wild type animals where only *2-Cys Prx* expression was increased (Figure 4d).

Parasite invasion into RBCs

In the absence of infection no significant difference in the percentage of mature RBCs or immature RBCs (reticulocyte) within the total RBCs was observed between α-TTP knockout and wild type mice (Figures 5a-c). However, throughout infection the percentages of infected reticulocytes (IRtc) in the total infected RBCs were significantly higher ($p < 0.05$) in α-TTP knockout mice than wild type animals (Figures 5b-d). These results support the notion that parasites in knockout mice tend to invade newly produced cells rather than mature RBCs, likely to evade the increased oxidative stress derived from vitamin E deficiency. This is in contrast to parasites in wild type mice that preferentially invaded mature RBCs during the acute phase of infection, and afterwards displayed a tendency to invade immature RBCs, probably due to a decrease in the number of mature RBCs that were available (Figure 5b). Taken together it appears that a high oxidative stress environment of knockout mice RBC leads to *Plasmodium* parasites to a preferential invasion of immature RBCs.

Effect of chloroquine administration coupled with α-TTP disruption

The possibility of combining α-TTP inhibition with existing malaria treatment in the form CQ treatment was examined [19]. When wild type mice were treated with 5 mg/kg of CQ their survival to *P. berghei* NK65 infection was significantly improved (Figure 6a), however, parasitaemia continued to increase beginning at day 9 and mice succumbed eventually to malaria by day 23 (Figures 6a-b). In contrast, identical treatment of α-TTP knockout mice resulted in 100% survival. Strikingly, these mice displayed undetectable levels of parasitaemia (Figures 6c-d). These results indicate that a combination of *α-TTP* inhibition and CQ administration could potentially be used as a useful treatment for malarial infection.

Discussion

Previous reports have indicated that reduction of host Vitamin E concentration through dietary restriction negatively impacted the development of *Plasmodium* and

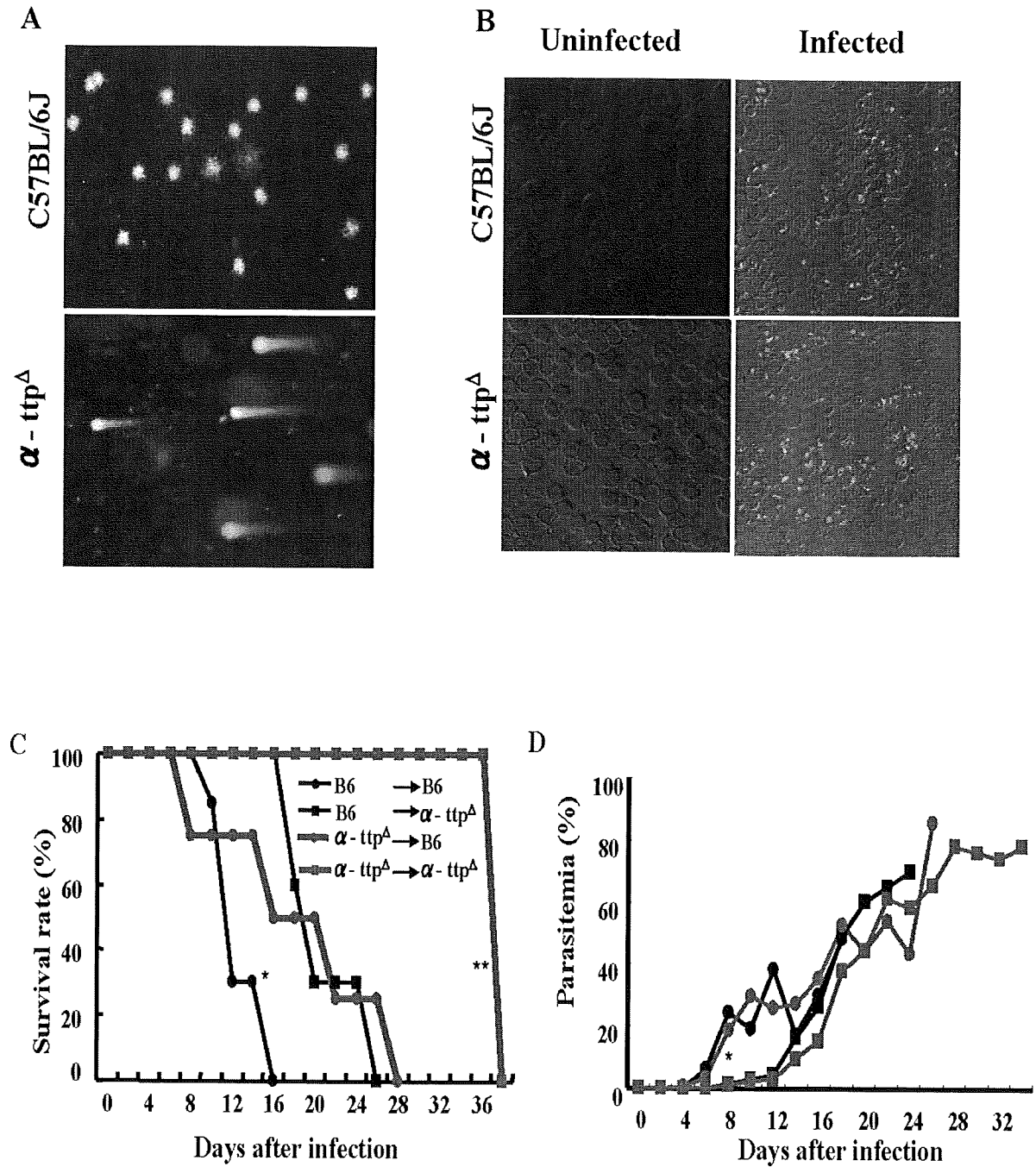

Figure 3 Vitamin E deficiency leads to *P. berghei* NK65 DNA damage. (A) Comet assay, indicating severe DNA damage, on the parasites recovered from α-TTP knockout or C57BL/6J mice. (B) Malarial parasites from α-TTP knockout and C57BL/6J mice stained with anti 8-OHdG (green), a biomarker of oxidative DNA damage. The DNA of the parasites was stained with propidium iodide (red) (n = 4). (C) C57BL/6J mice infected with parasites recovered from the α-TTP knockout (α-ttp$^{\Delta}$ T B6) or C57BL/6J mice (B6 T B6). α-TTP knockout mice infected with parasites recovered from α-TTP knockout mice (α-ttp$^{\Delta}$ T α-ttp$^{\Delta}$) or C57BL/6J mice (B6 T α-ttp$^{\Delta}$) (n = 6). *: $p < 0.05$ B6 T B6 vs α-ttp$^{\Delta}$ T B6; **: $p < 0.01$ B6 T α-ttp$^{\Delta}$ vs α-ttp$^{\Delta}$ T α-ttp$^{\Delta}$in panel C. In panel D *: $p < 0.05$ B6 T B6, α-ttp$^{\Delta}$ T B6 vs B6 T α-ttp$^{\Delta}$, α-ttp$^{\Delta}$ T α-ttp$^{\Delta}$.

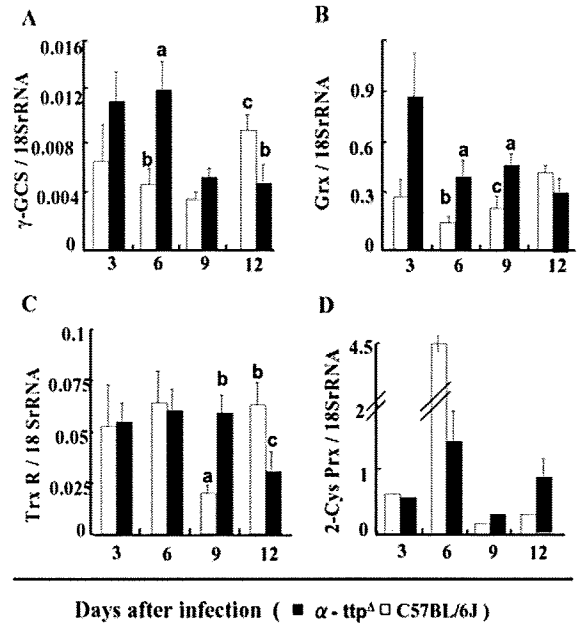

Figure 4 Expression of anti-oxidative enzymes in parasites. mRNA expression of glutaredoxin (Grx), γ-glutamylcysteine synthetase (γ-GCS), 2-Cys peroxiredoxins (Prx) and thioredoxin reductase (TrxR) of infected parasites in the C57BL/6J (white bar) and α-TTP knockout mice (black bar). Bars represents ± SEM; Bars not sharing common letters are significantly different ($p < 0.05$) by Scheffe multiple comparison test. (n = 6).

malaria development [11,12,20]. From 1954 to 1971, the notion that malnutrition was protective against malaria infection was promoted. This idea was strongly supported by epidemiological studies made from 1975 to 1980 by Murray *et al* [11]. In addition, animal studies appeared to support this idea. For example, Levander *et al* [21] demonstrated that vitamin E and selenium status have a profound impact on the ability of the host to resist acute infectious diseases. The relation between nutritionally-induced oxidative stress and malaria was firstly studied using the Chinese traditional anti-malarial drug, Qinghaosu. This compound is thought to destroy the parasite by generating oxy-free radicals. Therefore, the idea of dietary deprivation of vitamin E, a free radical scavenger, might be expected to enhance the anti-malarial activity of the drug [21]. In this study, rather than reduce vitamin E via diet, the α-TTP gene responsible for regulation of host vitamin E concentration was inhibited. Modification of host nutritional status through inhibition of α-TTP and subsequent lowering of vitamin E concentration conferred resistance to malarial infection (Figure 1).

Parasite proliferation was dramatically inhibited in the knockout mice infected with *P. yoelii* compared to the knockout mice infected with *P. berghei* NK65. The increased sensitivity to oxidative damage of *P. yoelii* compared to *P. berghei* has been demonstrated [22]. In these studies, mice infected with *P. yoelii* and treated with intravenous injection of H_2O_2 almost cleared parasites compared to those infected with *P. berghei* in which parasitaemia still increased. This phenomenon was due to an earlier and higher production of O_2 in the splenic macrophages as compared with the mice infected with *P. berghei*. Moreover, mice infected with *P. yoelii* sporozoites showed a decrement in the cellular inflammatory response against infection [23]. Therefore, it is speculated that vitamin E deficiency enhances the susceptibility of *P. yoelii* to oxidative damage due to the exacerbative production of ROS from the immune response of the host and likely due to a decreased immune response. Moreover, α-TTP knockout mice exposed to 10-20 *P. berghei* ANKA infected mosquitoes displayed similar survival rates to the α-TTP knockout mice infected by IRBCs via intraperitoneal injection (Additional file 1). Thus, it seems that *α-TTP* gene disruption does not influence development of the sporozoite stage of parasites in the liver (pre-erythrocytic stage). It is well known that vitamin E, through its free radical scavenging properties, prevents damage of molecules such as DNA, lipids and proteins in the face of production and biotransformation of ROS in biological systems [24]. Consistent with the notion that malaria resistance in α-TTP knockout mice resulted from lost vitamin E scavenging activity, reduced *Plasmodium* proliferation was reversible upon vitamin E supplementation (Figure 2). The absence of vitamin E in the host cells would lead to an oxidative stressful environment that would require parasites to efficiently use their anti-oxidant systems for survival such as the thioredoxin system [25-27].

Furthermore, anti-oxidant activity in these parasites was raised and DNA damage was evident. After initial high levels of expression the transcription of *γ-GCS*, *Grx* and *TrxR* genes dropped in the knockout mice, while parasitaemia increased presumably as the parasites adapted to the oxidative environment [28]. This is in contrast to parasites hosted within wild type mice where the expression of *γ-GCS* and *Grx* increased over time, as haemoglobin consumption would be expected to occur and consequently create an oxidative environment [29] (Figures 3a-b). Taken together, it appears that inhibition of *α-TTP* gene activity leads to an inhospitable environment for parasites residing within the host. Since the nature of dietary fat alters the lipid composition of RBC membranes and malarial parasites can not biosynthesize their own fatty acids, likely, parasites and RBC membranes of mice fed with vitamin E deficient diets enriched with fish oil containing diets are highly susceptible to peroxidation due to a normal production of ROS [20]. In this study, knockout mice fed with normal diet of vitamin E did not show early after infection stressing that one of the rea-

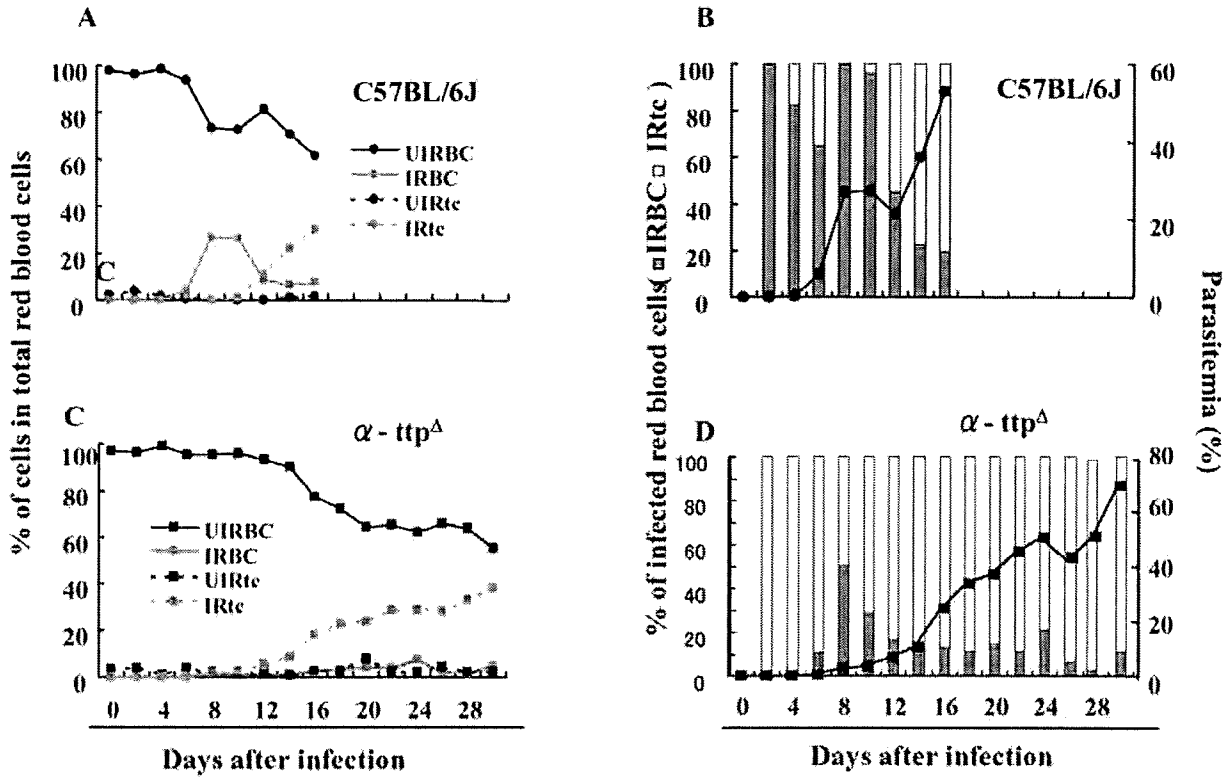

Figure 5 Parasites infecting α-TTP knockout mice intend to invade immature RBCs. Percentages of uninfected mature RBCs (UIRBC), infected mature RBCs (IRBC), uninfected reticulocytes (UIRtc), and infected reticulocytes (IRtc) from total RBCs of C57BL/6J in panel A and α-TTP knockout mice in panel C. Percentages of infected mature RBCs (gray bar) and infected reticulocytes (white bar) from total infected RBCs of C57BL/6J in panel B and α-TTP knockout mice in panel D (n = 6).

sons of inhibition of parasite proliferation in the knockout mice is clearly related to oxidative damage of the parasite. This suggests that knockout mice might possess additional mechanisms to keep normal levels of vitamin E in the RBC membranes [30]. It is impressive that α-TTP knockout mice survived with rising levels of parasitaemia at the later stage of the infection. Possible explanation might be that hosts surviving initial infection selected parasites less able to cause death to hosts. However, it is important to point out that whilst parasitaemia increases, anaemia status is more severe, suggesting that anaemia is a defense mechanism of the host for the prevention of parasite proliferation. Also, it can be speculated that parasites at this point are less virulent that the ones during the acute phase of the infection. Evidence for this notion comes from Comet assay revealing DNA damage to parasites hosted within the α-TTP knockout mice at the earliest points after infection. Furthermore, parasites recovered from these mice were less virulent than those recovered from the wild type mice; indeed, wild type mice infected with knockout recovered parasites survived significantly longer than those infected with wild type recovered parasites (Figures 3c-d). While these finding potentially explain the survival of knockout mice despite high levels of parasitaemia, they do not explain the observation of wild type mice surviving initial parasitaemia. This phenomenon, however, is certainly worth future study and may be used to gain insight into similar dynamics in human populations with malaria.

In this study, the mRNA expression of IL-10, INF-γ and TNF-α in liver, kidney, and spleen throughout infection was similar in the knockout mice as compared to the wild type mice suggesting that the inflammatory-immune response of the knockout mice is not altered by *α-TTP* disruption (manuscript in preparation). It has been suggested that the acquired immune response of the host likely play an important role in the parasite elimination [20]. When α-TTP knockout mice were infected with *P. yoelii* and re-infected with *P. berghei* ANKA on day 14 post-infection, 80% of the knockout mice were able to survive showing a very low parasitaemia. In contrast, 20% of the knockout mice infected with *P. berghei* ANKA or *P. yoelii* and *P. berghei* ANKA simultaneously were able to survive, indicating that knockout mice might be able to generate an acquired immunity or produce malarial antibodies against parasites. In this study parasite infecting

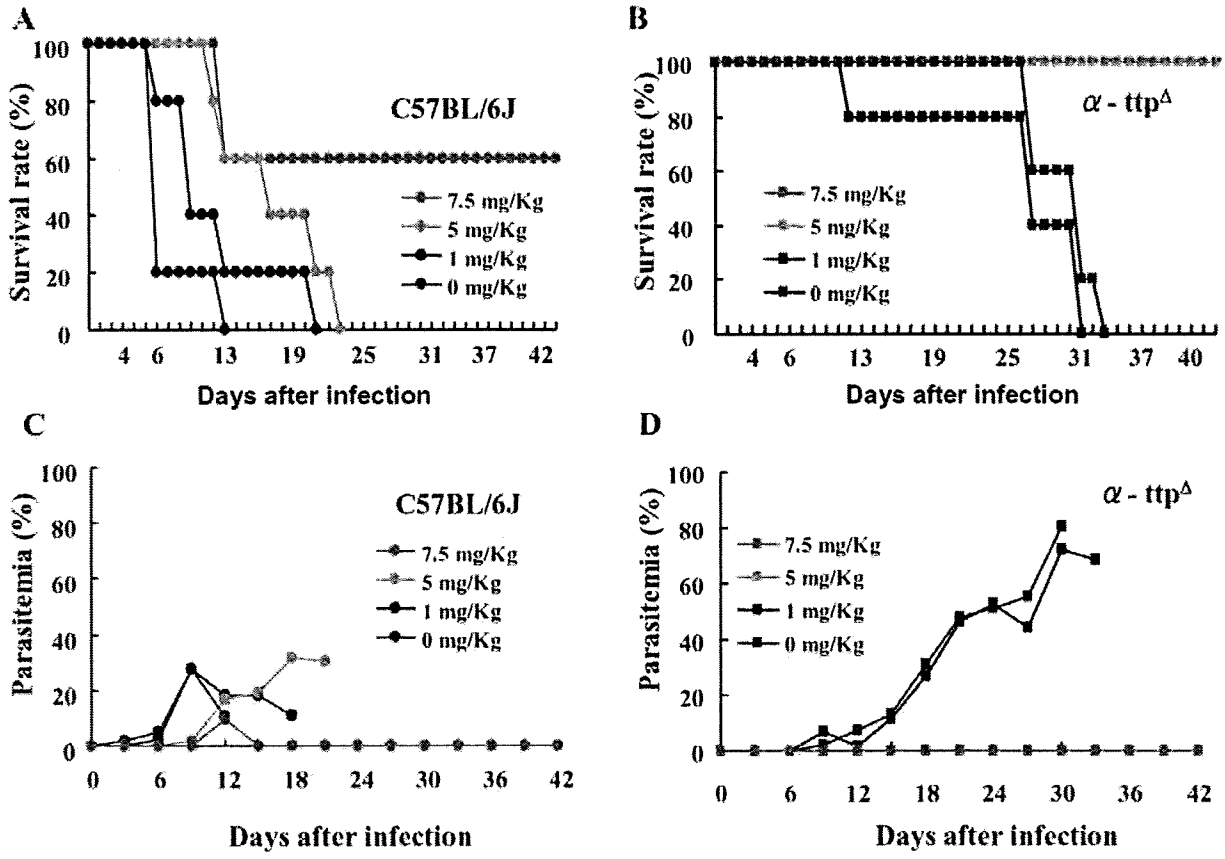

Figure 6 Chloroquine administration in combination with α-TTP disruption inhibits parasite proliferation. Mice were infected with *P. berghei* NK65 (4 × 10^5 IRBCs) and administered 0-7.5 mg/Kg of chloroquine on days 0, 1, and 2 after infection (n = 6).

the knockout mice were able to proliferate from day 15 after infection suggesting that either parasites might develop a resistance characteristic against malarial antibody or host protective immune response is impaired in these mice due to a mechanism of adaptation between parasite and mice. The mRNA expression of *IL-10, INF-γ*, and *TNF* in liver, kidney, and spleen throughout infection was similar in the knockout mice as compared to the wild type mice.

With the finding that inhibition of α-TTP leads to malaria resistance, potential exploitation as a novel therapeutic strategy becomes possible. Interestingly, inhibition of *α-TTP* did not trigger development of anaemia as indicated by the finding that no significant differences in RBC/reticulocyte ratio or total numbers of RBCs could be seen between the α-TTP knockout and wild type mice in uninfected mice (Figure 5a). Moreover, RBCs from the α-TTP knockout were normal in appearance and quality, the maximum and minimum resistance to osmotic stress at different concentrations of NaCl were the same (Additional file 2). Lastly, the concentration of reduced glutathione, an indicator of oxidative status, in the liver unchanged 19.3 ± 1.28 and 17.0 ± 1.20 for the C57BL/6J and α-TTP knockout mice, respectively (Additional file 3). Either α-TTP knockout mice absorb vitamin E from their diet to maintain transiently acceptable levels of vitamin E within the RBCs, as has been demonstrated in patients with familial isolated vitamin E deficiency [30], or other anti-oxidants such as ascorbate and β-carotene compensate for missing vitamin E in RBC membranes of α-TTP knockout mice [24]. Based on these observations it appears that α-TTP gene inhibition is a potentially valid therapeutic strategy.

Another prerequisite of *α-TTP* gene disruption as a potential therapeutic strategy lies in the ability to chemically inhibit the protein. Fortunately, in humans, a binding pocket in α-TTP, specific for α-tocopherol, has been revealed by crystallography and is considered to be responsible for the homeostasis of vitamin E [31]. Disruption of α-TTP inhibits α-tocopherol transfer from the liver and thus, would serve as an ideal target in drug design.

One of the most exciting findings from this study lies in the use of combinations of CQ [19], a classic anti-malarial drug, and gene disruption; levels of CQ found to leave parasites remaining in wild type mice removed all traces of the parasite from α-TTP knockout mice. By using such a combination, levels of CQ known to be toxic to patients could be avoided. Furthermore, drug resistance of *Plasmodium*, a serious problem in many parts of the world, could be avoided in a manner similar to avoidance of HIV drug resistant strains through combinations of drugs [32]. In terms of genetics, this duel approach is similar to synthetic lethality whereby simultaneous disruption of two genes results in a synergistic effect that is greater than either single gene disruption [32-34]. The finding that the presence of CQ is able to impair the intracellular alpha tocopherol transport suggests that CQ treatment and disruption of *α-TTP* likely are impinging on the same cellular process and therefore prone to such a synthetic therapeutic approach [35]. This synthetic lethal approach has been used recently for drug discovery [36] and has begun to be exploited in cancer research [37,38]. Lastly, it is possible that this type of strategy might be extended to other parasites as α-TTP gene disruption was found to be protective against a variety of parasites [39].

Conclusion

Inhibition of α-TTP activity in host leads to a resistance characteristic against malaria infection due to parasite's DNA oxidative damage. Therefore, α-TTP inhibition in host might be a new strategy for its prevention and control. Moreover, a combined strategy of α-TTP inhibition and chloroquine treatment might be effective against drug resistant parasites.

Additional material

Additional file 1 Survival rates of α-TTP knockout mice infected with *P. berghei* ANKA via mosquitoes or IRBCs. α-TTP knockout mice exposed to 10-20 P. berghei ANKA infected mosquitoes displayed similar survival rates to the α-TTP knockout mice infected by IRBCs via intraperitoneal injection.

Additional file 2 The maximum and minimum resistance to osmotic stress at different concentrations of NaCl in RBCs from the α-TTP knockout mice. The maximum and minimum resistance to osmotic stress at different concentrations of NaCl in RBCs from the α-TTP knockout mice were similar to those from wild type mice.

Additional file 3 Reduced glutathione concentrations of the liver in α-TTP knockout and wild type mice. Reduced glutathione concentrations of the liver were similar in α-TTP knockout and wild type mice.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MSH designed, conducted, analysed the data and wrote the manuscript, YYU conducted the experiments, CI conducted the experiments, MC conducted the experiments, KI conducted the experiments, MS advised for the vitamin E concentration measurements, SF conducted the experiments, NY conducted the experiments, MT analysed the data, XX analysed the data, HA analysed the data, HS conceived and designed the study and contributed to the preparation of the manuscript. All authors have read and approved the final manuscript.

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Author Details

[1]Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13, Inada, Obihiro, 080-8555 Japan, [2]Health Technology Research Center, National Institute of Advanced Industrial Science and Technology, Ikeda, Osaka, 563 Japan, [3]Department of Cell Pathology, Graduate School of Medical Science, Kumamoto University, 1-1-1, Honjo, Kumamoto, 860-8556 Japan, [4]Graduate School of Pharmaceutical Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033 Japan and [5]The United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu City, 501-1193 Japan

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Functional Identification of the *Plasmodium* Centromere and Generation of a *Plasmodium* Artificial Chromosome

Shiroh Iwanaga,[1,*] Shahid M. Khan,[2] Izumi Kaneko,[1] Zoe Christodoulou,[3] Chris Newbold,[3] Masao Yuda,[1] Chris J. Janse,[2] and Andrew P. Waters[2,4,*]
[1]Mie University, School of Medicine, Tsu 514-0001, Japan
[2]Leiden Malaria Research Group, Centre of Infectious Diseases, Leiden University Medical Centre, Leiden 2333 ZA, The Netherlands
[3]Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DS, UK
[4]Present address: Division of Infection and Immunity, Institute of Biomedical Life Sciences and Wellcome Centre for Molecular Parasitology, Glasgow Biomedical Research Centre, University of Glasgow, Glasgow G12 8TA, Scotland
*Correspondence: iwanaga@doc.medic.mie-u.ac.jp (S.I.), waters@bio.gla.ac.uk (A.P.W.)
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SUMMARY

The artificial chromosome represents a useful tool for gene transfer, both as cloning vectors and in chromosome biology research. To generate a *Plasmodium* artificial chromosome (PAC), we had to first functionally identify and characterize the parasite's centromere. A putative centromere (*pbcen5*) was cloned from chromosome 5 of the rodent parasite *P. berghei* based on a *Plasmodium* gene-synteny map. Plasmids containing *pbcen5* were stably maintained in parasites during a blood-stage infection with high segregation efficiency, without drug pressure. *pbcen5*-containing plasmids were also stably maintained during parasite meiosis and mitosis in the mosquito. A linear PAC (L-PAC) was generated by integrating *pbcen5* and telomere into a plasmid. The L-PAC segregated with a high efficiency and was stably maintained throughout the parasite's life cycle, as either one or two copies. These results suggest that L-PAC behaves like a *Plasmodium* chromosome, which can be exploited as an experimental research tool.

INTRODUCTION

The centromere in eukaryotic cells plays a fundamental role in the fidelity of chromosome segregation during nuclear division, through its physical association with the kinetochore. Centromere-associated functions include sister chromatid association and separation, microtubule attachment, chromosomal movement, and establishment of heterochromatin and mitotic checkpoint control (Cleveland et al., 2003; Morris and Moazed, 2007; Pluta et al., 1995). The centromere was first identified in the budding yeast *Saccharomyces cerevisiae*: a plasmid containing a yeast centromere was demonstrated to segregate stably during both mitosis and meiosis, providing experimental evidence for the function of the centromere (Clarke and Carbon, 1980). Following this functional identification, a linear DNA construct, including the centromere, telomeric DNA from *Tetrahymena* rDNA termini, and the autonomously replicating sequence, was generated and termed yeast artificial chromosome (YAC) (Murray and Szostak, 1983). YACs can be stably maintained in a linear form in cells during nuclear division and are widely utilized as a vector for transferring large DNA fragments in yeast (Burke et al., 1987). Several artificial chromosomes for other eukaryotes have since been constructed using this approach of combining the same three elements (Harrington et al., 1997; Ikeno et al., 1998). These artificial chromosomes are being used not only as gene-transfer and cloning vectors but also as genetic tools in chromosome function/biology research (Grimes et al., 2004; Okada et al., 2007).

The goal of this study was to generate a *Plasmodium* artificial chromosome (PAC) as a tool to assist in genome and genetic studies. To achieve this goal, we had first to functionally identify and characterize the centromeres of *Plasmodium*. Putative centromeres (PCENs) of the unicellular protozoan parasite *P. falciparum* were previously identified as 2–3 kb A/T-rich regions with no protein-coding potential, based on a sequence comparison between two *P. falciparum* chromosomes (Bowman et al., 1999). After completion of the genome sequence of *P. falciparum*, PCEN regions with similarly high A/T-rich content (>96%) were described for 13 of the 14 chromosomes (Gardner et al., 2002). Further comparisons of *P. falciparum* chromosomes with a whole-genome synteny map of three rodent malaria parasites (RMPs) revealed a high level of synteny between the genomes of different *Plasmodium* species that extends to the location of the PCEN (Kooij et al., 2005). A more recent study located PCENs on *P. falciparum* chromosomes by examining the distribution of etoposide-mediated topoisomerase-II cleavage sites (Kelly et al., 2006). The PCEN locations identified by this study agreed entirely with the locations predicted by whole-genome sequencing and confirmed the presence of A/T-rich domains with a strict size range of 2.3–3.5 kb. Although these studies have provided an insight into sequences and locations of PCENs, experimental evidence is lacking as to whether these highly A/T-rich DNA regions alone can function as *Plasmodium* centromeres. Based on the syntenic location of PCEN, we identified and cloned the PCEN of *P. berghei* chromosome 5

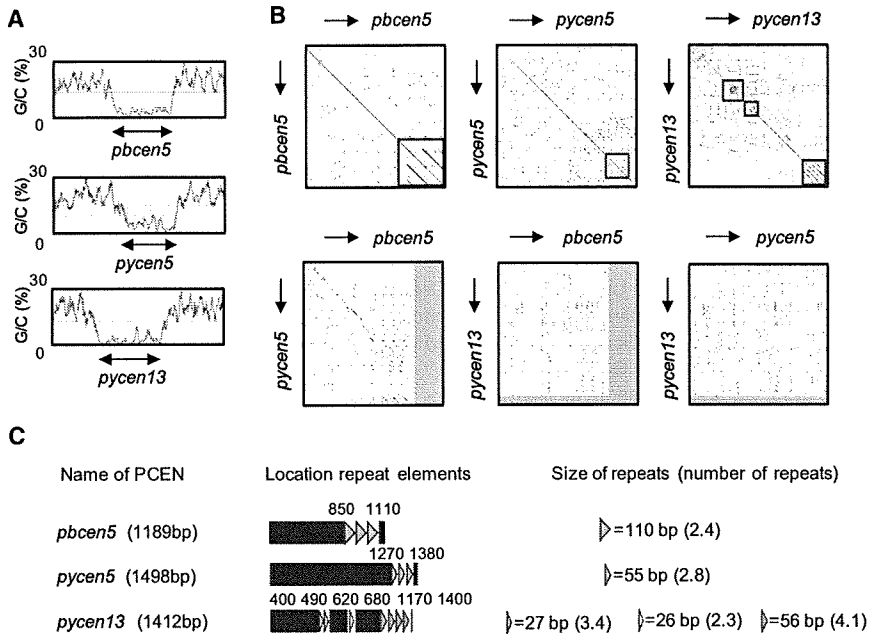

Figure 1. PCENs Are Highly A/T Rich and Consist of Core and Repetitive Regions

(A) The DNA AT content of centromeric regions of *P. berghei* chromosome 5 and *P. yoelii* chromosomes 5 and 13 was analyzed using Artemis 10. Arrows indicate the ultra-high A/T-rich regions termed PCENs (*pbcen5*, 1189 bp; *pycen5*, 1498 bp; and *pycen13*, 1412 bp).

(B) Dot matrix analysis of the three rodent malaria PCENs using the Dotlet program. In this analysis, only the PCEN regions identified in (A) were used. Panels depict the graphical results of the matrix analysis of the rodent PCENs aligned either against themselves or against the other rodent PCENs. The diagonal line within each analysis represents sequence identity, and parallel lines to the diagonal indicate repetitive regions within each PCEN. Boxes highlight repetitive regions used for further analysis. See also Figure S1A.

(C) Lengths and numbers of sequence motifs in the repetitive regions (boxes in B) identified in the dot matrix analyses were determined using the Tandem Repeats Finder program (see also Figure S2 and Table S1). Based on these analyses, the locations, lengths, and numbers of repetitive elements are schematically depicted for the three rodent PCENs analyzed. Each triangle represents a repetitive sequence motif/element. See also Figure S1B and Table S1.

(*pbcen5*) in plasmids. We show that plasmids containing *pbcen5* were both efficiently and stably maintained in transfected parasites during blood-stage multiplication, without drug pressure, demonstrating that *pbcen5* can confer the function of a *Plasmodium* centromere.

In order to generate a PAC, we combined both *pbcen5* and *Plasmodium* telomere sequences into a single plasmid. The third conventional element of a YAC, autonomous replication sequences, has not been identified in *Plasmodium*. However, much *Plasmodium* research has involved plasmid transfections, and despite the varying degrees of segregation efficiency of these plasmids into daughter parasites, they have all been able to replicate inside the parasite, indicating that a variety of DNA sequences can act as *Plasmodium* origins of replication. Telomere sequences of *P. berghei* have been previously identified and cloned (Pace et al., 2000; Ponzi et al., 1985). Evidence has been found that telomere lengthening occurs in *Plasmodium* chromosomes mediated by the activity of *Plasmodium* telomerase enzyme (Figueiredo et al., 2005; Pace et al., 2000; Ponzi et al., 1992). Here we provide evidence that a linear construct, L-PAC, containing both the identified centromere, *pbcen5*, and telomere sequences behaves like a *Plasmodium* chromosome. It segregates with high efficiency during mitosis and meiosis, all in the absence of drug selection pressure, and is stably maintained during the complete life cycle as a few (unconcatemerized) copies. Moreover, we find evidence for telomere elongation, in keeping with the L-PAC being maintained and recognized as a *Plasmodium* chromosome. We discuss the possibilities of using PACs in *Plasmodium* research and how it promises to significantly expand the currently limited set of genetic modification techniques available to understand and manipulate the parasite.

RESULTS

Cloning and Sequence Properties of Putative *Plasmodium* Centromeres

In our previous study, PCENs from chromosomes 5 and 13 of *P. yoelii* were cloned by PCR amplification based on the gene-synteny map of RMP (Kooij et al., 2005). DNA fragments of 4.5 and 4.1 kb were obtained from chromosomes 5 and 13, respectively, which included highly A/T-rich regions predicted as PCENs: *pycen5* (DQ054838.1, 1498 bp, A/T = 97.7%) and *pycen13* (DQ054839.1, 1412 bp, A/T = 98.1%) (Figure 1A). In this study, we amplified the PCEN of chromosome 5 from *P. berghei*. For this PCR we used the same primer pair as was used for *pycen5*, because there were no available sequences from the regions proximate to the PCENs of *P. berghei* based on the RMP gene-synteny map (see the Supplemental Experimental Procedures available online). The amplified 3.8 kb DNA fragment included a highly A/T-rich region, and thus it was predicted to be *pbcen5* (i.e., GU809989, 1189 bp and A/T content of 96.1%; Figure 1A).

To investigate the sequence properties of *pbcen5*, a dot matrix analysis was performed using the Dotlet program (Figure 1B and see also the Supplemental Experimental Procedures). In this analysis, a strict threshold was applied to determine sequence identity, as the sequence complexity of PCEN was low due to its high A/T content. Identity was only recorded if the sequences within a 15 bp sliding box were more than 80% identical. The dot matrix analysis showed that *pbcen5* included a repetitive region indicated by parallel lines in Figure 1B. Furthermore, similar analyses of *pycen5* and *pycen13* as well as 13 annotated PCEN regions from *P. falciparum* (sequence information for PFCEN on chromosome 10 is absent) showed that these PCENs contained

one to three repetitive regions. These results suggest that PCENs consist of the nonrepetitive region, termed the "core region," and the repetitive region, and this general sequence organization is conserved between different *Plasmodium* species (Figures 1B and 1C and Figure S1).

Reciprocal dot matrix analysis between various PCENs showed that sequence identity (indicated by the diagonal line) was found between *pbcen5* and *pycen5* (Figure 1B, the left panel in the lower row). A BLAST2 analysis between these two rodent malaria PCENs showed that they shared homology with a sequence identity of 79%. In contrast, no significant sequence identity was found in reciprocal analyses between the other rodent malaria and *P. falciparum* PCENs (data not shown). Interestingly, sequence identity between *pbcen5* and *pycen5* was restricted to the core regions, indicating that the rates of evolution of the core and the repetitive regions are different.

We further analyzed the possible existence of consensus repetitive motifs within the repetitive regions of the PCENs using the Tandem Repeats Finder program. As can be seen in Figure 1C, Figure S1, and Table S1, different lengths and numbers of repetitive motifs can be distinguished in the various PCENs; however, because of widely divergent sequences, no consensus repetitive repeats were detected (data not shown).

Segregation of Plasmids Containing a PCEN in *P. berghei* Transgenic Blood-Stage Parasites

During blood-stage asexual multiplication, *Plasmodium* chromosomes are segregated between daughter cells during a process termed schizogony, which differs in several aspects from mitosis in most other eukaryotes. After DNA replication, chromosome segregation occurs without observable chromosomal condensation or breakdown of the nuclear envelope (Aikawa, 1966; Rudzinska, 1969), resulting in polyploid syncytia after several (three to four) rounds of genome duplication. Only in the final step of mitosis when the individual daughter parasites (8–16 merozoites) are formed do the individual nuclei bud off from the syncytium. In contrast to this even segregation of chromosomes between the daughter cells, episomally maintained plasmids (introduced by transfection) segregate unevenly during schizogony, such that several of the daughter merozoites do not receive plasmids during each multiplication cycle (O'Donnell et al., 2001; van Dijk et al., 1997). This uneven distribution leads to a growth disadvantage and is believed to explain why plasmids are rapidly lost when transfected parasites are grown in the absence of drug pressure.

To determine whether *pbcen5* contributed to efficient segregation of episomal plasmids during schizogony, we examined the stability of a plasmid including *pbcen5* in blood-stage parasites without drug pressure. As shown in Figures 2B and 2C, the GFP expression in parasites transfected with the control pbGFPcon plasmid lacking PCEN was rapidly lost due to its uneven segregation. However, GFP expression remained stable in the parasites transfected with the plasmid including *pbcen5*, termed pbCEN5 (Figure 2A), and approximately 90% of the parasites with the pbCEN5 plasmid continued to express GFP 21 days after drug pressure had been removed (Figures 2B and 2C). During parasite blood-stage development, we found no growth disadvantage conferred by retaining the pbCEN5 plasmid, as compared to either wild-type or pbGFPcon-transfected parasites. This is based on the observation of comparable courses of parasitemia after infection with standard dose of 10^4–10^5 of either wild-type or PCEN-containing parasites. These results clearly demonstrate that the pbCEN5 plasmid can be stably maintained in parasites independent of drug treatment during multiplication, indicating that pbCEN5 segregated evenly into daughter parasites (cells) during asexual blood-stage mitosis.

Similar results were obtained when the stability of a plasmid containing the 4.5 kb DNA fragment including *pycen5* was assessed in the parasite. The percentage of GFP-positive parasites with pyCEN5 was comparable to that of pbCEN5 in the absence of drug pressure as described above (Figures 2B and 2C). Interestingly, the pfCEN3 plasmid was not as stably maintained in blood-stage *P. berghei* as the plasmids containing a centromere of RMP origin. The percentage of GFP-positive parasites decreased to <60% within 21 days (Figures 2B and 2C), although it was higher than that of parasites transfected with the control plasmid pbGFPcon.

To confirm that the PCEN plasmids pbCEN5, pyCEN5, and pfCEN3 were retained as episomes and not integrated into the parasite genome during nuclear replication, we analyzed the genomic DNA of the parasites collected at the start and end of the 18–21 day multiplication period. HindIII-digested DNA was hybridized with a *Pbdhfr-ts* 5′UTR DNA fragment, as this probe recognizes both the endogenous *dhfr-ts* gene (4.9 kb fragment) and the plasmid. As seen in Figure 2D, only the endogenous *dhfr-ts* gene and the plasmid were detected in all parasite populations, both at the beginning and at the end of the multiplication period, indicating that the plasmids were not integrated into the parasite genome but were maintained in episomal form in the parasite nuclei. These results were supported by plasmid rescue experiments in which we were able to recover intact plasmids from all of the parasite populations (data not shown).

Using the percentages of GFP-expressing parasites at the end of the 18–21 day period, we calculated segregation efficiencies for each of the plasmids, based on the assumption that during schizogony the parasite nuclei undergo four nuclear divisions, resulting in the production of 16 daughter nuclei over a 24 hr period (see the Experimental Procedures). The segregation efficiencies of the plasmids pbCEN5, pyCEN5, pfCEN3, and pbGFPcon were calculated as 99.9%, 99.9%, 99.3%, and 93.9% per nuclear division, respectively. When the predicted percentages of GFP-positive parasites were plotted on the basis of the calculated segregation efficiencies over time, it was seen that these percentages fit closely with the observed percentages of GFP-positive parasites (Figure 2C and Figure S2).

Southern analysis of plasmid presence indicated that in parasites maintained in the presence of pyrimethamine, the hybridization signal of the pbGFPcon plasmid was significantly stronger than that of the PCEN plasmids (Figure 2D), indicating that the former plasmid is maintained at a higher number of copies in *P. berghei* (Figure 2D). Comparison of signal intensities for DNA extracted from parasites transfected with the various plasmids and maintained under drug pressure revealed that the pbGFPcon plasmid copy number is on average 28.3 (SE ± 0.3; Table 1), whereas the PCEN plasmid copy numbers are significantly lower (i.e., pbCEN5, 8.0 ± 0.4; pyCEN5, 6.6 ± 0.3; and pfCEN3, 5.4 ± 1.6 copies per parasite; Table 1). After

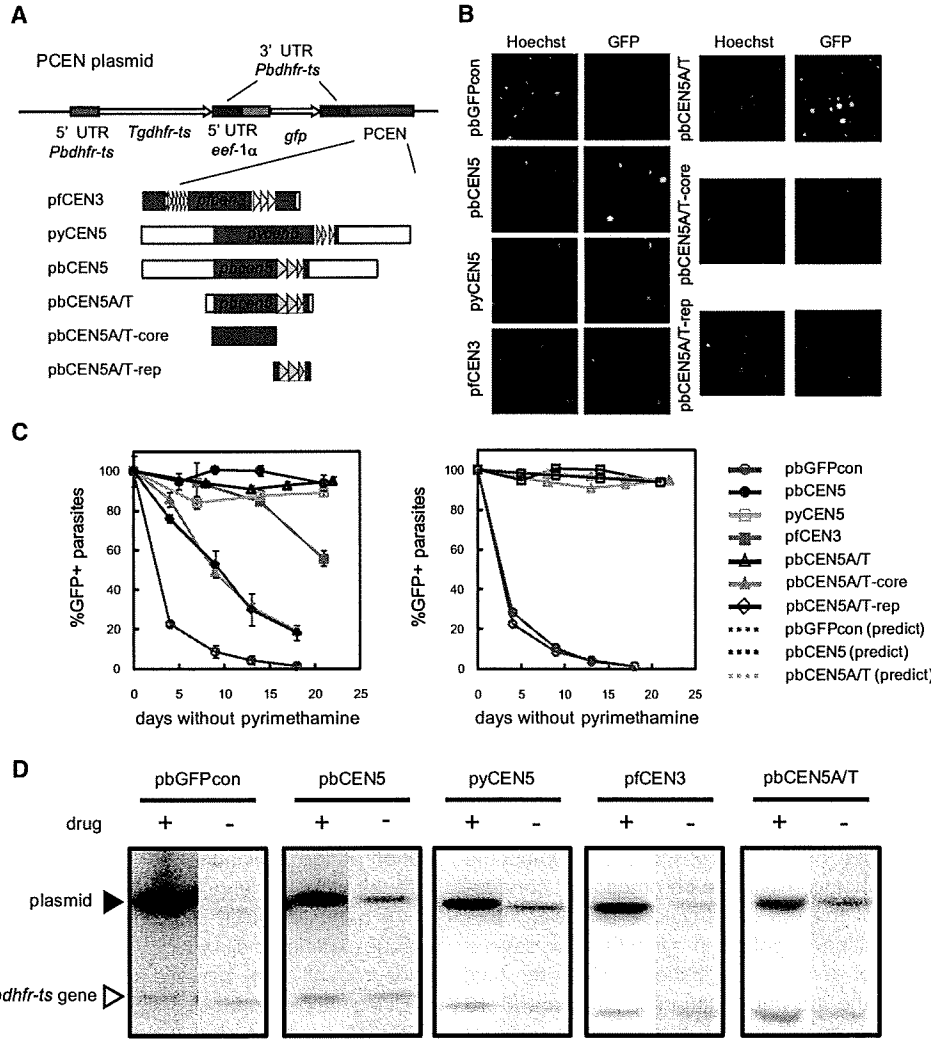

Figure 2. Addition of PCEN to Plasmids Improves Their Segregation Efficiency during *P. berghei* Blood-Stage Multiplication

(A) Schematic representation of the various PCEN plasmids. The plasmid contains the *Tgdhfr-ts* selectable marker and the GFP reporter protein under the control of the *eef1αa* promoter. pbGFPcon is a control plasmid without a PCEN. The names of the resulting PCEN plasmids are indicated to the left of the schematic drawings of the cloned PCEN DNA fragments.

(B) GFP expression of Hoechst 33258-stained (all parasites) blood-stage *P. berghei* transfected with the various PCEN plasmids, 18–21 days after drug withdrawal.

(C) The percentage of GFP-positive parasites during asexual multiplication of blood-stage *P. berghei* that have been transfected with the various PCEN-containing plasmids (left panel). Observed percentage (right panel) of GFP-positive parasites determined by fluorescence microscopy (solid lines) compared to the predicted percentage of GFP-positive parasites (dashed lines), based on the calculated segregation efficiencies of the various plasmids (see also Figure S2). The error bars represent standard error.

(D) Southern analysis of the presence of plasmids in the parasites either after 7 days of multiplication in the presence of pyrimethamine (+) or after 18–21 days in the absence of pyrimethamine (–). Blots were hybridized to the *P. berghei* 5′UTR *dhfr-ts* probe, which recognizes the plasmid (black triangle) and the endogenous single *dhfr-ts* genome copy (white triangle, 4.9 kb). The sizes of signals from each plasmid are as follows: pbGFPcon, 9.0 kb; pbCEN5, 12.4 kb; pyCEN5, 13.1 kb; pfCEN3, 11.5 kb; and pbCEN5A/T, 10.4 kb.

removal of drug pressure, the PCEN plasmid copy number 21 days later was reduced by 2- to 4-fold for the RMP centromere-containing plasmids and >5-fold for pfCEN3. However, there was a strong reduction (>47-fold) in the pbGFPcon plasmid copy number (Table 1). These results indicate that the PCEN elements not only confer greater segregation efficiency to the plasmids but also maintain their low copy number independent of drug treatment.

Only the Highly A/T-Rich Centromeric Sequences Are Required to Confer High Segregation Efficiency to Plasmid in Blood-Stage Parasites

We next examined whether only the highly A/T-rich region (consisting of both the core and repetitive regions) predicted to be *pbcen5* was sufficient to produce high segregation efficiency. First, the amplified 1.4 kb fragment including *pbcen5* (1189 bp) was cloned into the plasmid, thereby creating the plasmid

Table 1. The Copy Number of PCEN Plasmids and PAC in Parasites

	+Drug	−Drug
pbGFPcon	28.3 (0.3)	0.6 (0.1)
pbCEN5	8.0 (0.4)	1.9 (0.1)
pyCEN5	6.6 (0.3)	2.8 (0.3)
pfCEN3	5.4 (1.6)	0.9 (0.1)
pbCEN5A/T	2.1 (0.3)	1.3 (0.4)
C-PAC	1.8 (0.6)	1.3 (0.1)
L-PAC	2.1 (0.6)	1.3 (0.1)

Copy numbers of PCEN plasmids, C-PAC and L-PAC were calculated from the ratio of intensities obtained from Southern analysis as shown Figures 2D and 5D. The standard errors are indicated in the parenthesis.

pbCEN5A/T (Figure 2A). The pbCEN5A/T plasmid was introduced into *P. berghei*, and its stability during mitosis in the absence of drug pressure was examined. Indeed, pbCEN5A/T was stably maintained during mitosis of asexual blood-stage parasites similar to the pbCEN5 plasmid, indicating that only the A/T-rich region (i.e., *pbcen5*) was required to confer high segregation efficiency to episomal plasmids (99.9%) (Figures 2B and 2C). Southern analysis using DNA isolated from the transfected parasites showed that pbCEN5A/T was maintained as an episomal plasmid throughout parasite multiplication and, further, that a low copy number was maintained both in the presence and absence of the drug (Figure 2D, Table 1). These results demonstrate that plasmids containing only the highly A/T-rich region, consisting of the core and repetitive regions, behave essentially the same as the other RMP PCEN plasmids.

To determine the minimal functional element of *pbcen5*, we generated two additional plasmids that contained either only the core (nonrepeat) or only the repeat sections of the A/T-rich region of *pbcen5*, designated as pbCEN5A/T-core and pbCEN5A/T-rep, respectively (Figure 2A). In parasites transfected with these plasmids, the percentage of GFP-expressing blood-stage parasites gradually decreased in the absence of drug pressure (Figures 2B and 2C): segregation efficiencies were 97.7% for pbCEN5A/T-core and 97.3% for pbCEN5A/T-rep. These efficiencies were higher than that of the control pbGFPcon plasmid but lower than those of the plasmids containing the complete A/T-rich region. These results indicate that the entire A/T-rich region is the minimal functional unit of *pbcen5* necessary to permit efficient segregation and maintenance of plasmids during asexual blood-stage mitosis.

Plasmids Containing PCEN Elements Are Stably Maintained in Parasites during Meiosis and Mitosis inside the Mosquito

In addition to asexual multiplication during blood-stage development, malaria parasites have a sexual phase involving meiosis (within the zygote/ookinete) and additional phases of extensive asexual multiplication in the mosquito host (within the oocysts). During meiotic and mitotic division of parasites in the mosquito host, segregation of chromosomes also occurs without chromosome condensation or breakdown of the nuclear membrane, resulting in polyploid nuclei (polyploid syncytia) containing as few as 4 (i.e., the tetraploid ookinete, after meiosis) to as many as 10,000 haploid genomes (i.e., midgut sporozoites). To analyze the segregation behavior of the PCEN plasmids during meiosis and mitosis in the mosquito, parasites carrying pbCEN5, pbCEN5A/T, or pbGFPcon were transmitted to mosquitoes, and the GFP expression of their salivary gland sporozoites was examined. The results indicated that 89.2% ± 3.6% and 89.9% ± 3.3% of sporozoites infected with parasites carrying pbCEN5 and pbCEN5A/T plasmids, respectively, expressed GFP, whereas only 1.9% ± 1.0% of sporozoites with parasites carrying the pbGFPcon plasmid were GFP positive (Figure 3). A growth disadvantage to parasites carrying either of the PCEN plasmids was not observed, suggesting that retention of these plasmids did not affect on the development of parasites in the mosquito. These results showed that addition of *pbcen5* sequences to plasmids greatly improves segregation and

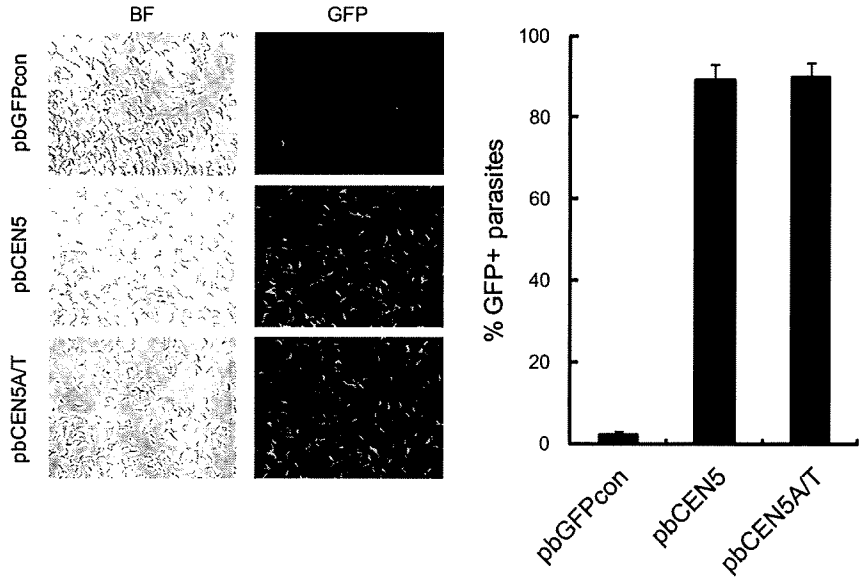

Figure 3. Retention of PCEN-Containing Plasmids in Parasites after Mosquito Passage

(Left panel) The GFP expressions in salivary gland sporozoites containing pbCEN5, pbCEN5A/T, or the control pbGFPcon are shown (BF, bright field image of the same sporozoites). (Right panel) The percentages of GFP-positive sporozoites (right) for parasites transfected with the various PCEN-containing plasmids were determined by fluorescence microscopic analysis. The error bars represent standard error.

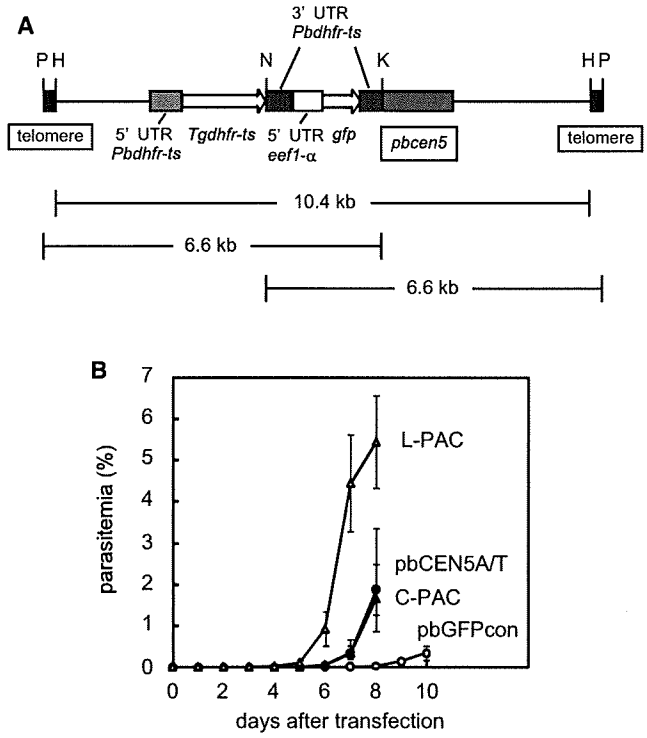

Figure 4. Transfection of Parasites with the Linear *Plasmodium* Artificial Chromosome Construct, Containing PCEN and Telomere Sequences

(A) Schematic representation of the L-PAC construct. The construct contains the *Tgdhfr-ts* selectable marker and the GFP reporter protein under the control of the *eef1αa* promoter. Restriction sites for PmeI (P), HindIII (H), NheI (N), and KpnI (K) are shown, which were used for the Southern analysis to determine the size of the DNA fragments digested with each enzyme.

(B) The course of parasitemia in mice infected with pbGFPcon (open circle), pbCEN5A/T (closed circle)-, L-PAC (open triangle)-, and C-PAC (closed triangle)-transfected parasites. All parasites (1×10^8) were transfected with 5 μg DNA, and mice were treated with pyrimethamine 1 day after i.v. injection of parasites. The error bars represent standard error.

maintenance during both meiosis in zygotes/ookinetes and mitosis in oocysts.

Generation of a *Plasmodium* Artificial Chromosome

The results reported above clearly indicate that the region of highly A/T-rich DNA predicted to be a *Plasmodium* centromere did indeed confer the function of a centromere to plasmids during parasite nuclear division. We therefore attempted to construct a PAC consisting of this *pbcen5* centromere as well as *P. berghei* telomeric DNA sequences. Briefly, a DNA insert containing two telomeric fragments, consisting of the previously characterized *P. berghei* telomeric CCCT(A/G)AA sequences (Pace et al., 1987) oriented head to head and separated by an ~500 bp spacer region, was cloned into the pbCEN5A/T plasmid, resulting in a plasmid designated circular *Plasmodium* artificial chromosome (C-PAC). The linear DNA construct (L-PAC) was generated through linearization of the C-PAC construct using PmeI, which removes the spacer fragment between the two telomeric fragments (Figure 4A). Unexpectedly, the transfection efficiency of the L-PAC was significantly higher than that of the circular plasmids. In multiple independent experiments, the mice injected with L-PAC-transfected parasites developed an parasitemia of ~1% 6 days after transfection, whereas a parasitemia of ~1% level was not observed until day 7 or 8 in mice injected with parasites transfected with the same amount of circular plasmid DNA, i.e., the C-PAC or pbCEN5A/T (Figure 4B).

The *Plasmodium* Artificial Chromosome Is Stably Maintained throughout the Complete Life Cycle

Segregation and maintenance of the C-PAC and the L-PAC constructs were investigated using the same methodologies as described for the PCEN plasmids. Approximately 85% of the C-PAC- and L-PAC-transfected blood-stage parasites expressed GFP 20 days after withdrawal of drug pressure (Figures 5A and 5B), and their segregation efficiencies at the blood stage were calculated as 99.8% and 99.9%, respectively. To confirm that neither the C-PAC nor the L-PAC was integrated into the parasite genome during replication, Southern analysis was carried out using KpnI-digested genomic DNA for the C-PAC and undigested genomic DNA for the L-PAC. A single 11.5 kb fragment was detected in the KpnI-digested genomic DNA for the C-PAC (Figure 5D), and a single fragment was detected in the undigested genomic DNA for the L-PAC (Figure 6, lanes 5 and 9), demonstrating that neither PAC was integrated into the genome. A similar analysis using HindIII-digested genome DNA for the C-PAC and the L-PAC determined copy numbers of 1.8 ± 0.6 and 2.1 ± 0.6 in drug-treated parasites, respectively, and 1.3 copies for both PAC vectors in the absence of drug treatment (Figure 5D and Table 1). These efficiencies of segregation and copy numbers in the blood stage were comparable to those of the pbCEN5A/T plasmid without the telomeric sequences. Next, we analyzed maintenance of the C-PAC and the L-PAC during mosquito-stage development and found that 83.2% ± 1.6% and 85.5% ± 1.6% of the sporozoites expressed GFP, respectively (Figures 5B and 5C). All of these results demonstrate that the addition of telomeric sequences and linearization did not alter the segregation efficiency and maintenance observed for plasmid pbCEN5A/T during mitosis and meiosis, suggesting that the centromere is able to function within either a circular or a linear DNA construct.

We also examined segregation of the C-PAC and the L-PAC in the dividing forms in the liver. After sporozoites are injected by a mosquito, they migrate to the liver, where they invade hepatocytes. It is in the hepatocytes where they undergo a rapid expansion: one infected hepatocyte can produce several thousand merozoites. For this analysis, it was not possible to estimate the segregation efficiency directly in liver cells; therefore, we assessed the percentage of GFP-positive (i.e., containing either L- or C-PAC) blood-stage parasites as soon as they were patent in the blood after liver stage development. As mentioned above, ~85% of the PACs-containing sporozoites were GFP positive, and the percentage of GFP-positive infected red blood cells after liver stage development was 62.2% ± 3.4% and 71.2% ± 3.4% for C-PAC and L-PAC, respectively (Figures 5B and 5C). The patent period in mice after infections established with sporozoites containing either C- or L-PAC was comparable to the patent period after infections initiated with the same number of wild-type sporozoites (data not shown). Those results would

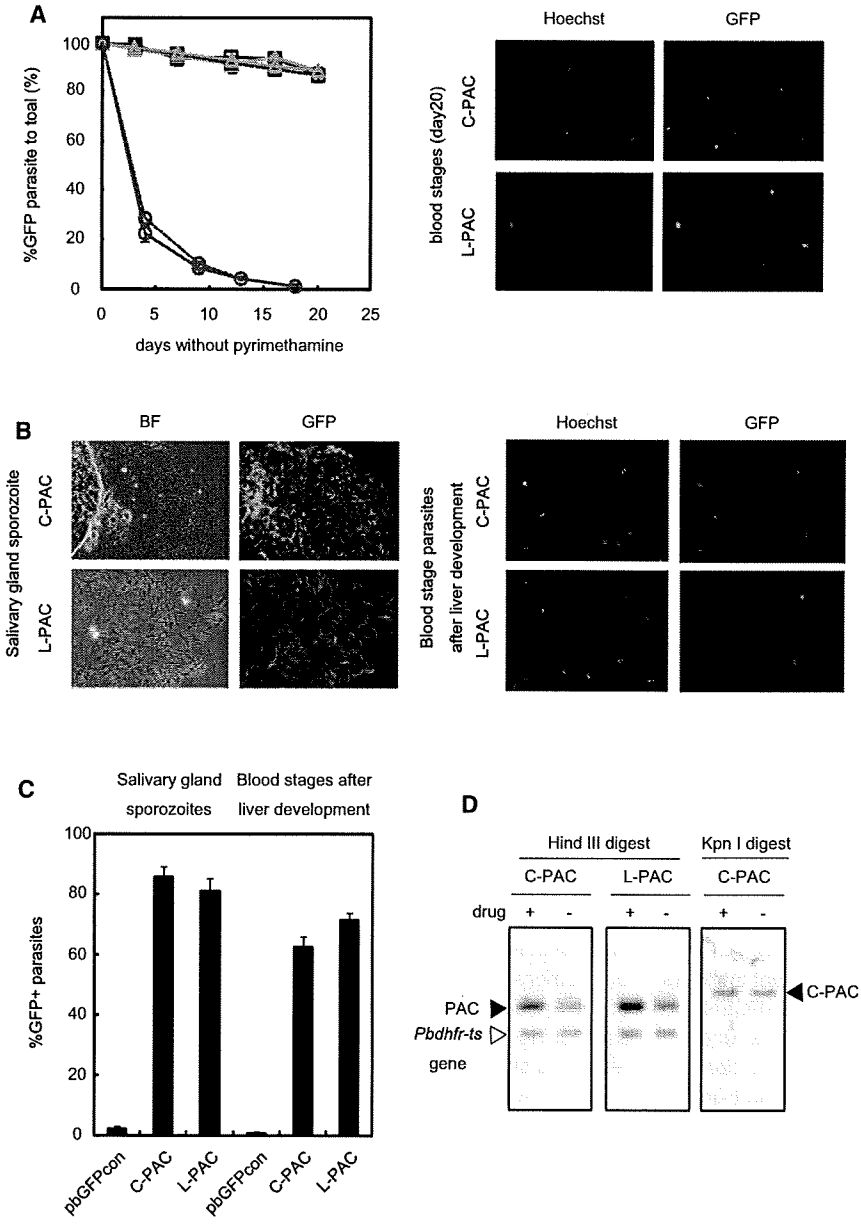

Figure 5. Efficient Segregation and Maintenance of Circular and Linear DNA Constructs Containing Both *pbcen5* and Telomere Sequences

(A) (Left panel) The percentages of GFP-positive parasites during blood-stage asexual multiplication of *P. berghei* transfected with the C-PAC (blue), L-PAC (green), or the control plasmid pbGFPcon (red) are shown. The observed percentages of GFP-positive parasites are determined by fluorescence microscopy (solid lines) compared to the predicted percentages of GFP-positive parasites (dashed lines), based on the calculated segregation efficiencies of the various constructs. The error bars represent standard error. (Right panel) The GFP expressions (C-PAC- and L-PAC-containing parasites) of Hoechst 33258-stained transfected parasites (all parasites) were observed by fluorescence microscopy after 18–20 days of asexual multiplication in the blood, without drug pressure.

(B) (Left panel) The GFP expressions in salivary gland sporozoite of parasites transfected with the C-PAC or the L-PAC are shown (BF, bright field image of the same sporozoites). (Right panel) GFP expression in the blood stages of parasites transfected with the C-PAC and L-PAC after first completing a passage through the mosquito and multiplication in the liver are shown.

(C) Percentage of GFP-positive, variously transfected (C-PAC, L-PAC, and pbGFPcon) salivary gland sporozoites after parasite development in the mosquito, as well as the percentage of GFP-positive blood stages after parasite passage through multiplications in the mosquito and in the liver. The error bars represent standard error.

(D) Southern analysis showing the presence of the DNA constructs in blood-stage parasites after either 7 days of multiplication in the presence of pyrimethamine (+) or 20 days in the absence of pyrimethamine (–). Southern analysis was performed on HindIII-digested C-PAC- or L-PAC-transfected parasite DNA with *P. berghei* 5′UTR *dhfr-ts* as a probe, which recognizes both the construct (black triangle) and the endogenous *dhfr-ts* genome copy (white triangle). Based on this result, the copy numbers are estimated and given in Table 1. Southern analysis of C-PAC-transfected parasite DNA digested with KpnI using the *gfp* gene as a probe identified the presence of a single fragment, indicating that the C-PAC is not integrated into the parasite genome.

indicate that despite the rapid expansion in parasite numbers in the liver, plasmids segregated with a high efficiency during the multiple rounds of mitotic division during liver-stage development.

To confirm whether the L-PAC was as an independent linear DNA construct throughout the complete parasite life cycle, we analyzed genomic DNA obtained from blood-stage parasites before and after mosquito transmission. The results of Southern analysis of undigested DNA hybridized with a probe against *gfp* demonstrated that the L-PAC is maintained as an extrachromosomal DNA construct during all stages of the parasite's life cycle (Figure 6). Southern hybridization with the same probe on the same DNA but now digested with the "single cutting" restriction enzymes, KpnI or NheI, revealed only a single band (~7 kb), which is again consistent with a linear, nonintegrated construct. Interestingly, both in the blood stages after 20 days of multiplication and after mosquito transmission, the size of both the KpnI and the NheI fragments was slightly larger than the expected 6.6 kb fragment of the original L-PAC. Moreover, the total size of the undigested construct was also slightly larger (~12 kb) than the size of the original L-PAC (10.8 kb). However, the internal fragment of the L-PAC remaining after HindIII digestion, which does not contain the telomeric sequences, was the same size as the original L-PAC. These results would therefore indicate that the increase in size of the undigested construct and the increase in the KpnI- or NheI-restricted fragments results from

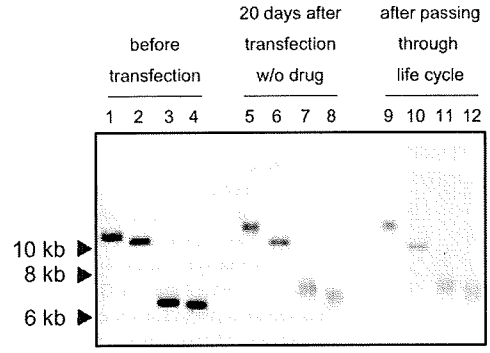

Figure 6. The L-PAC Was Maintained as a Linear Form in the Parasite throughout the Complete Life Cycle

Southern analysis to examine the L-PAC construct in transfected parasites after 20 days of multiplication in the blood in the absence of pyrimethamine, as well as in the re-emerging parasite in the blood after mosquito passage and multiplication in the liver. Genomic DNA was digested with HindIII (lanes 6 and 10), KpnI (lanes 7 and 11), or NheI (lanes 8 and 12) and hybridized with the *gfp* gene. In addition, undigested genomic DNA (lanes 5 and 9) was also resolved. Undigested L-PAC (lane 1) and HindIII (lane 2)-, KpnI (lane 3)-, and NheI (lane 4)-digested L-PAC were analyzed under the same conditions.

an increase in size of the two telomeric fragments, indicating that telomere lengthening occurs by addition of telomeric repeats and, further, that the L-PAC telomeres were maintained at a length of up to 1.4 kb.

DISCUSSION

After completing the sequencing of the *P. falciparum* genome, PCENs were predicted to be within 2–3 kb highly A/T-rich (>96%) gene-free regions. However, despite the recent increase in knowledge about the sequence and location of PCEN, no functional evidence demonstrating that PCEN regions function as centromeres has been reported. In this study, we demonstrated that the A/T-rich region predicted to be the centromere of chromosome 5 of *P. berghei* (*pbcen5*) conferred improved segregation efficiency to episomal plasmids. Transfected plasmids containing *pbcen5* were stably maintained in parasites in the absence of drug pressure. In *Plasmodium*, standard plasmids (e.g., without PCEN elements) are rapidly lost from transfected parasites during asexual multiplication when no drug selection is applied. The stable maintenance of the *pbcen5*-containing plasmids provides strong evidence that the *pbcen5* does indeed function as centromere and that these data constitute the functional characterization of a *Plasmodium* centromere.

The PCEN regions consist of a nonrepetitive region (core) and regions containing repetitive elements, as identified by dot matrix analysis. In an attempt to define a "minimal functional unit" within the *pbcen5* centromere, we analyzed the maintenance of plasmids that contained either only the regions containing repetitive elements or the core sequence. While some increase in segregation efficiency of plasmids is derived from having either one of these elements, high segregation efficiencies were only achieved when both the PCEN repeat and core regions are present, indicating that the entire A/T-rich sequence (i.e., both the core and the repetitive regions) is required for functioning of a centromere. Dot matrix analyses of the other available PCENs from *P. falciparum* and *P. yoelii* indicated a similar organization of all PCENs, consisting of the core and repetitive regions. Despite this conservation of the overall PCEN structure, we did not find conservation in location, sequence, or number of repeats between different chromosomal PCENs, neither within the same genome nor between different species. These results are in large part in agreement with centromere analyses of *P. falciparum* performed by Kelly et al. (Kelly et al., 2006). These observations suggest that the sequence organization and DNA composition of PCEN regions are important to centromere function.

In most strains of *Saccharomyces cerevisiae*, a 6.3 kb plasmid is present, the so-called "2 μm plasmid," which is replicated by a "rolling-circle" mechanism and is maintained in high copy numbers (50–100 copies/cell) (Broach and Hicks, 1980; Tschumper and Carbon, 1983). The addition of a yeast centromere to the 2 μm plasmid suppresses its rolling-circle replication, resulting in maintenance of only a few copies of the plasmid (one to five copies per cell) (Tschumper and Carbon, 1983). Standard *Plasmodium* plasmids, such as pbGFPcon, also replicate through a rolling-circle mechanism, and these ultimately form large concatameric multimers generated by both intra- and intermolecular recombination. In the present study, plasmids containing *pbcen5* are maintained in low copy numbers (i.e., one to two copies per cell) during blood-stage multiplication, indicating that *pbcen5* also prevents the formation of concatamers by suppressing a rolling-circle replication by the addition of a centromere.

The linear L-PAC construct, containing both the *pbcen5* and telomeric sequences, is stably maintained throughout the complete life cycle of the parasites, indicating that the addition of telomere sequences prevented the degradation of the ends of the linear construct. Attempts to generate transgenic parasites using a linear construct containing *pbcen5* but without telomeric sequences were unsuccessful (data not shown), supporting the importance of telomeric sequences in the maintenance of a linear construct. Our Southern analysis of L-PAC DNA obtained from parasites after successive rounds of replication in both host and vector indicates a lengthening of "telomeric ends" of the L-PAC plasmid and their maintenance within a certain length (1.4 kb). In *P. berghei*, telomere lengthening occurred after the introduction of a short (~500 bp) telomeric sequence at the end of a chromosome by a targeted terminal deletion event, resulting in a telomere extension to about a length of 1.2 kb (Pace et al., 2000), which is the average length of *P. berghei* telomeres (Dore et al., 1994). This excellent concordance between our present result and previous study suggested that telomere elongation and regulation of its length in L-PAC occurred by the same mechanism observed in parasite original chromosome.

In *P. falciparum*, multigene families encoding clinically important proteins such as the variant antigens PfEMP-1, RIFIN, and STEVOR are located in subtelomeric regions of chromosomes (Gardner et al., 2002). Genes of these multigene families are often expressed in a mutually (or partially mutually) exclusive manner (Scherf et al., 1998). Recent ChIP-on-chip analyses have demonstrated that the Lys9 of histone H3 is trimethylated