Brief Definitive Report

Transfection of the Primate Malaria Parasite *Plasmodium knowlesi* Using Entirely Heterologous Constructs

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Summary

The recently developed transfection systems for *Plasmodium berghei* and *Plasmodium falciparum* offer important new tools enabling further insight into the biology of malaria parasites. These systems rely upon artificial parasite–host combinations which do not allow investigation into the complex interactions between parasites and their natural hosts. Here we report on stable transfection of *Plasmodium knowlesi* (a primate malaria parasite that clusters phylogenetically with *P. vivax*) for which both natural and artificial experimental hosts are available. Transfection of this parasite offers the opportunity to further analyze the biology of antigens not only in a natural host but also in hosts that are closely related to humans. To facilitate future development of integration-dependent transfection in *P. knowlesi*, completely heterologous plasmids that would reduce homologous recombination at unwanted sites in the genome were constructed. These plasmids contained the pyrimethamine-resistant form of dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) from *Toxoplasma gondii* or *P. berghei*, under control of either (*a*) *P. berghei* or (*b*) *P. falciparum* promoters. Plasmids were electroporated into mature *P. knowlesi* schizonts and these cells were injected into rhesus monkeys (*Macaca mulatta*). After pyrimethamine treatment of these monkeys, resistant parasites were obtained that contained the plasmids. Promoter regions of both *P. berghei* and *P. falciparum* controlling *dhfr-ts* expression were effective in conferring pyrimethamine resistance in *P. knowlesi*, indicating that common signals control gene expression in phylogenetically distant *Plasmodium* species.

The recent development of systems for the stable transformation of malaria parasites offers the prospect of genetic approaches to the understanding of the biology of the parasites (1–6). It is anticipated that these approaches will find valuable application in the development of vaccines and new drugs. To date, stable transfection of the human parasite *P. falciparum* (2) and the rodent parasite *P. berghei* (3) has been achieved through the introduction of plasmids carrying the gene encoding the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (*dhfr-ts*), either obtained from *Plasmodium* species or from *Toxoplasma gondii* (7), as selectable marker. Under the control of conspecific and homologous promoter and downstream regions, this gene conferred resistance against the anti-malarial pyrimethamine.

Although recognizing the value of transfection for the study of *P. berghei* and *P. falciparum*, these parasites do not easily allow investigations of interactions between parasites and their natural host. The rodents available for infection with *P. berghei* are phylogenetically distant from the natural host and the few animal models susceptible to *P. falciparum* infection (new world monkeys and chimpanzees) are unnatural hosts and have infection characteristics distinct from the human host.

Here we report on the stable transfection of the primate malaria parasite *P. knowlesi*, a parasite for which both the natural and artificial vertebrate hosts are available, offering the possibility to study the biology of antigens in a natural host–parasite combination and in hosts that are closely related to the human host. In addition, as a result of the substantially different infection characteristics of *P. knowlesi* in the natural (*Macaca fascicularis*) and the closely related artificial host (*Macaca mulatta*) it also provides an ideal opportunity to further our understanding of the mechanisms of immunity to malaria (8). An additional advantage of *P. knowlesi* is that a considerable investment has already been made in the analysis of antigens of this parasite (for example see references 9–14), among which important analogues exist in human malaria parasites.

A powerful aspect of transfection is the possibility for site-specific integration of DNA into the genome by ho-

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mologous recombination, which allows the functional analysis of specific molecules through targeted disruption or modification of genes. With a view to developing an integration-dependent transfection system for *P. knowlesi*, in this study we used constructs that contained both (*a*) entirely heterologous selection markers and (*b*) control regions from *P. berghei* and *P. falciparum* to reduce recombination at unwanted sites of the genome, assessing whether these parasites, although phylogenetically distinct from *P. knowlesi*, may have signals in common with *P. knowlesi* that control gene expression.

Materials and Methods

DNA Constructs. Plasmid pDT.Tg23 and pchD5.1/C3 have previously been described (5, 6). Plasmid pD.D_B.D. contains the same elements as pMD204 (3) except that the selection cassette was cloned into pUC-19 for pD.D_B.D. instead of pBluescript, and the elements (upstream, ORF and downstream) were engineered so that the ORF is readily replaceable through excision with BamHI. The pyrimethamine-resistant M2M3 mutant form of the *T. gondii dhfr-ts* gene (7) was amplified from pDT.Tg23 by PCR, using primers 5′-CGTGATCAATGCATAAAACCGGTGTGTC-3′ (TOX3) and 5′-CGTGATCAAAGCTTCTGTATTTCCGC-3′ (TOX4). PCR with *pfu* polymerase (Stratagene Inc., La Jolla, CA) yielded an amplified product that was kinated, gel-purified, and cloned into the blunted BamHI site of plasmid pD.D_B.D. (replacing the *P. berghei dhfr-ts*) to yield pD.D_T.D. Plasmids were purified using Plasmid Mega columns (Qiagen, Chatsworth, CA).

Parasite Manipulations. A *P. knowlesi* (Nuri strain) (15) infection was initiated in a female rhesus monkey (*Macaca mulatta*) by intravenous injection of 1×10^5 parasites. Parasitemia was monitored daily on blood obtained from finger pricks. When 35% of erythrocytes were infected with mature schizonts, blood was collected by cardiac puncture. After centrifugation (450 *g*, 10 min, room temperature) the top brown layer of the erythrocyte pellet, containing >90% schizonts, was collected. Leukocytes were removed from this material using PlasmodiPur filters (Eurodiagnostica, Apeldoorn, the Netherlands) (16). Schizonts were then suspended in either PBS (3) or incomplete Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes, pH 7.6) (2) at a concentration of 5×10^9 schizonts/ml.

Transfection and Selection of Transformants. Two different plasmid mixtures were prepared: Mix 1 consisted of pD.D_B.D. and pD.D_T.D. (mixed 1:1 wt/wt) and Mix 2 consisted of pDT.Tg23 and pchD5.1/C3 (mixed 1:1 wt/wt). For each electroporation, a total of 100 μg plasmid of Mix 1 or Mix 2 was added to 5×10^8 schizonts in a 0.4-cm electroporation cuvette and electroporated using a Bio-Rad Gene Pulser using the following conditions (previously established for *P. falciparum* and *P. berghei* [2, 3]). DNA dissolved into 85 μl TNE (10 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 7.5) and 115 μl PBS was mixed with schizonts in the same buffer and electroporated at either 600, 800, or 1,200 V, at a capacitance of 25 μF (time constants ranged between 1.1–1.5 ms). DNA dissolved into 700 μl incomplete Cytomix was mixed with schizonts in Cytomix and subjected to a pulse of either 1,500, 2,000, or 2,500 V, at a capacitance of 25 μF and a resistance of 200 Ω (time constants ranged between 0.7–0.8 ms). Samples electroporated under these conditions were pooled, placed on ice for 5–8 min and injected intravenously into two non-splenectomized rhesus monkeys. Monkey R3106 received pooled electroporated samples of Mix 1 and monkey R3126 received pooled electroporated samples of Mix 2. Starting 40 h after injection of schizonts both monkeys orally received 2 mg/kg pyrimethamine per day, supplemented once a week with 3.5 mg folinic acid to counteract the bone marrow suppression caused by pyrimethamine (17). The parasitemia of the two monkeys was monitored daily. After 11 d of pyrimethamine pressure, blood was collected by cardiac puncture and leukocytes were removed by PlasmodiPur filtration. Parasite DNA was isolated and analyzed according to standard protocols.

Results and Discussion

Transformation of P. knowlesi with Heterologous Plasmids Yielded Pyrimethamine-resistant Parasites. Based on the successful use of mutated *dhfr-ts* genes conferring pyrimethamine resistance as selectable markers in transfection systems of *P. berghei* and *P. falciparum*, we transfected *P. knowlesi* with plasmid constructs containing resistant forms of the *dhfr-ts* gene of *P. berghei* or *T. gondii* (Table 1). 36 h after inoculation of transfected schizonts in monkey R3106 and R3126, newly invaded parasites were readily detectable in thin smears. After pyrimethamine treatment was initiated, parasitemias rapidly dropped to levels undetectable by thick-film analysis, confirming the sensitivity of *P. knowlesi* to this drug. However, under continuous pyrimethamine administration, parasitemias in both monkeys rose to detectable levels by day 8. On day 12, when >1% of the red blood cells were infected, the pyrimethamine-resistant parasites were collected for further analyses (Table 1).

Resistant Parasites Contained the T. gondii dhfr-ts Gene in Stable Episomal Form. To assess which genes and control regions are active in *P. knowlesi*, we separately introduced two different plasmid combinations into *P. knowlesi*. Monkey R3106 received schizonts transfected with Mix 1, containing plasmid constructs with *P. berghei dhfr-ts* or *T. gondii dhfr-ts* flanked in both cases by control regions of *P. berghei dhfr-ts*. Monkey R3126 received schizonts transfected with Mix 2 containing a plasmid construct with *T. gondii dhfr-ts* flanked by *P. falciparum* control regions and a non-selectable plasmid to control for the possibility of continued presence of plasmid which does not confer pyrimethamine resistance.

DNA was isolated from the parasites of monkeys R3106 and R3126 and hybridized with probes against *P. berghei dhfr-ts*, *T. gondii dhfr-ts* or *P. falciparum cam* (Fig. 1 *A*). Hybridization of DNA of parasites from both monkeys was evident with the *T. gondii dhfr-ts* probe, indicating that, consistent with findings in the *P. falciparum* and *P. berghei* systems (5, our unpublished observation), *T. gondii dhfr-ts* was effective in conferring pyrimethamine resistance to *P. knowlesi*. The plasmid containing *P. berghei dhfr-ts* was not detected by this analysis. However, PCR analysis of DNA from parasites of monkey R3106 was positive for *P. berghei dhfr-ts* (data not shown). The presence of only minor amounts of pD.D_B.D. compared with pD.D_T.D. might suggest that parasites containing *P. berghei dhfr-ts* are overgrown by parasites containing *T. gondii dhfr-ts*. In the *P. berghei* system *T. gondii dhfr-ts*

Table 1. *Plasmids Used in the Transfection of P. knowlesi and Resultant Parasite Development*

Rhesus	Plasmids	Selectable marker	Control regions	Parasitaemia		
				+ 36 h	+ 84 h	+ 12 d
					%	
R3106	pD.D_B.D. pD.D_T.D.	*P. berghei dhfr-ts* *T. gondii dhfr-ts*	*P. berghei dhfr-ts* *P. berghei dhfr-ts*	0.4	0	1.4
R3126	pDT.Tg23 pchD5.1/C3	*T. gondii dhfr-ts* *cat*	*P. falciparum hrp3/hrp2* *P. chabaudi dhfr-ts* *P. falciparum cam*	0.6	0	1.1

was found to confer a 10–100-fold higher pyrimethamine resistance to the parasites than *P. berghei dhfr-ts* (Janse, C.J., unpublished data). This may result in a selective advantage for parasites containing the *T. gondii dhfr-ts* under drug pressure, although other effects on growth kinetics, for example direct effects of the expression product of both plasmids cannot be ruled out.

The presence of plasmid pD.D_T.D. and plasmid pDT.Tg23 in resistant parasites was confirmed by rescue experiments through transformation of *E. coli* with parasite DNA. Unrearranged plasmids were recovered, as was shown by restriction analysis (Fig 1 *B*). We failed to rescue plasmid pD.D_B.D. and pchD5.1/C3.

To confirm that plasmids had been replicated in a eukaryotic environment, susceptibility to cleavage by MboI was evaluated (18). Plasmids isolated from *E. coli* were not susceptible to MboI digestion, but were susceptible to DpnI, an isoschizomer that is active when the adenine in the rec-

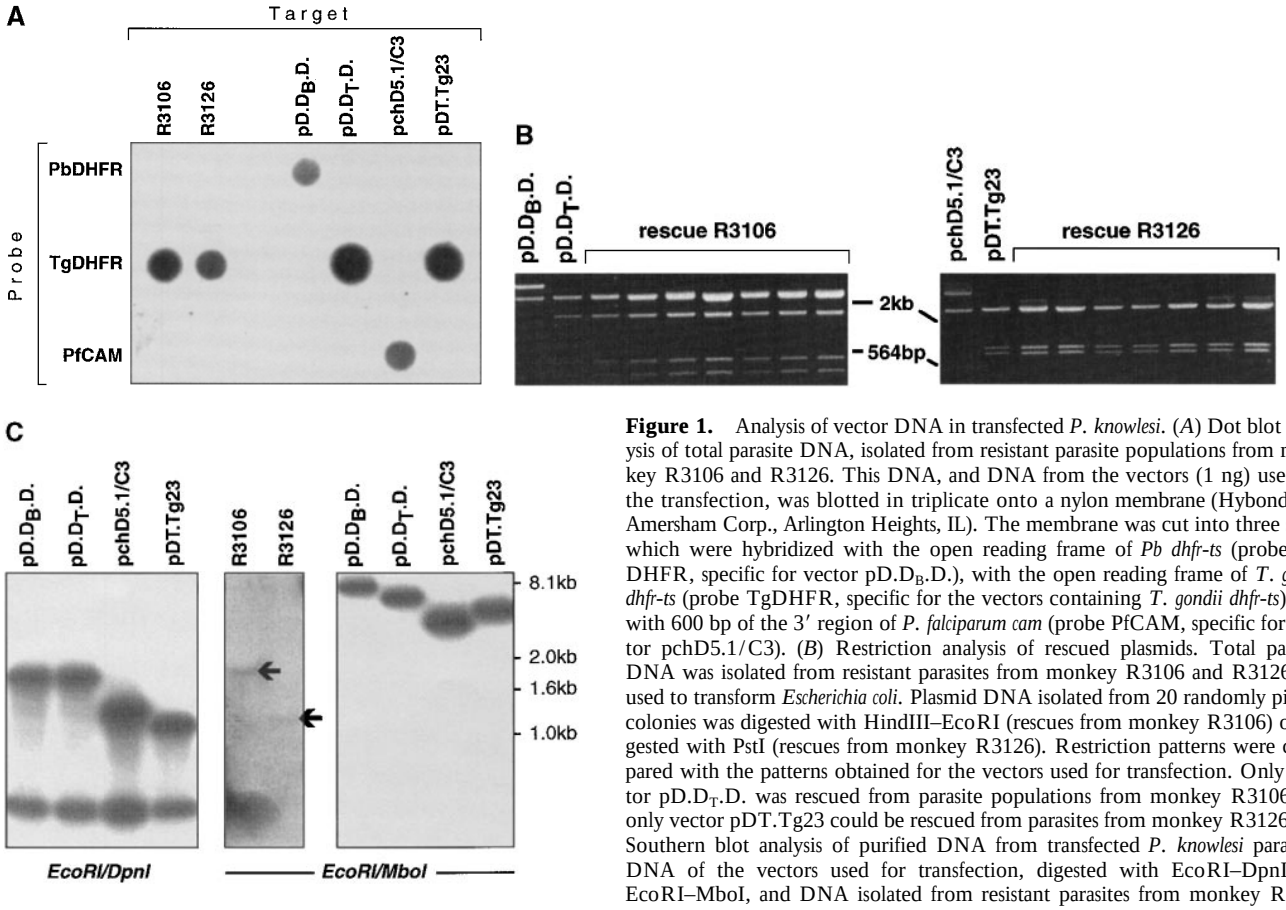

Figure 1. Analysis of vector DNA in transfected *P. knowlesi*. (*A*) Dot blot analysis of total parasite DNA, isolated from resistant parasite populations from monkey R3106 and R3126. This DNA, and DNA from the vectors (1 ng) used for the transfection, was blotted in triplicate onto a nylon membrane (Hybond N^+, Amersham Corp., Arlington Heights, IL). The membrane was cut into three strips which were hybridized with the open reading frame of *Pb dhfr-ts* (probe PbDHFR, specific for vector pD.D_B.D.), with the open reading frame of *T. gondii dhfr-ts* (probe TgDHFR, specific for the vectors containing *T. gondii dhfr-ts*), and with 600 bp of the 3′ region of *P. falciparum cam* (probe PfCAM, specific for vector pchD5.1/C3). (*B*) Restriction analysis of rescued plasmids. Total parasite DNA was isolated from resistant parasites from monkey R3106 and R3126 and used to transform *Escherichia coli*. Plasmid DNA isolated from 20 randomly picked colonies was digested with HindIII–EcoRI (rescues from monkey R3106) or digested with PstI (rescues from monkey R3126). Restriction patterns were compared with the patterns obtained for the vectors used for transfection. Only vector pD.D_T.D. was rescued from parasite populations from monkey R3106 and only vector pDT.Tg23 could be rescued from parasites from monkey R3126. (*C*) Southern blot analysis of purified DNA from transfected *P. knowlesi* parasites. DNA of the vectors used for transfection, digested with EcoRI–DpnI and EcoRI–MboI, and DNA isolated from resistant parasites from monkey R3106 and R3126, digested with EcoRI–MboI, was fractionated on a 0.6% agarose gel and blotted onto a nylon membrane (Hybond N^+). The blot was hybridized with a *pBluescript* PvuI–SacI restriction fragment and washed with 0.1% SSC, 0.1% SDS at 65°C.

ognition site is methylated, as occurs in prokaryotic systems (5, 18). Plasmids isolated from parasites from both monkeys were susceptible to MboI digestion, demonstrating their eukaryotic replication (Fig 1 *C*). Transcription of *T. gondii dhfr-ts* in *P. knowlesi* was shown by hybridization with a Northern blot containing RNA isolated from parasites of monkey R3106. A transcript with a size of 2.4 kb was detected (not shown), comparable to the size of the transcript produced in *P. berghei* by plasmid pD.D_B.D. (Tomás, A.M., unpublished data).

Considering the phylogenetic distance between *P. knowlesi*, *P. berghei*, and *P. falciparum* (19, 20) and the large differences in the GC-contents of the genomes of these species (18% for both *P. berghei* and *P. falciparum* and 30% for *P. knowlesi* [21]), it is of interest that control regions of both *P. falciparum* and *P. berghei* were functional (were able to drive expression of the *dhfr-ts* genes) in *P. knowlesi*. This finding may be related to specific characteristics of the genome composition of *P. knowlesi*. In a study using CsCl-density centrifugation it has been shown that the genomes of the closely related species *P. vivax* and *P. cynomolgi*, separate into high-density (GC-rich) and low-density (AT-rich) components. Although the genome of *P. knowlesi* contains only a high-density component (21), specific sequences of the *P. knowlesi* genome do hybridize with the low-density component of *P. cynomolgi*, indicating that AT-rich sequences are present in the *P. knowlesi* genome. Additionally, in a comparison of introns of different *Plasmodium* species, introns in both *P. vivax* and *P. knowlesi* were found to have either a GC-rich or an AT-rich composition (22). Introns are likely to reflect their genomic environment, and therefore, it was suggested that the differences in the introns of these genes reflect their maintenance in distinct isochores (very long DNA segments with fairly homogeneous base compositions [23]). This suggests that the genome organization of *P. knowlesi* may share the characteristics of both *P. vivax* and *P. cynomolgi*. If this is the case, transcription machinery in *P. knowlesi* may be able to respond to control regions of varied base composition, and this would explain why in *P. knowlesi*, gene control regions derived from phylogenetically distinct parasites with a different overall genomic GC-content are functionally active.

In summary, we have shown that *P. knowlesi* can be stably transfected using entirely heterologous constructs, offering a model system which allows investigations into parasite-host interactions, in hosts closely related to humans. The successful use of heterologous constructs in this parasite will facilitate the creation of transgenic and knockout parasites through integration-dependent transfection. The phylogenetic distance over which the control elements have been shown to be effective in this study suggests that similar constructs may also be effective in *P. vivax* and in other important non-human primate malaria parasites such as *P. cynomolgi* and *P. fragile*.

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