2001

Evidence for Different Mechanisms of Chloroquine Resistance in 2 *Plasmodium* Species That Cause Human Malaria

Takashi Nomura
Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases

Jane M-R Carlton
Computational Biology Branch, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda

J. Kevin Baird
US Naval Medical Research Unit No. 2, Jakarta, Indonesia, jkevinbaird@yahoo.com

Hernando A. del Portillo
Departamento de Parasitologia, Universidade de São Paulo, São Paulo, Brazil

David J. Fryauff
Malaria Program, Naval Medical Research Center, Silver Spring, Maryland

See next page for additional authors

Evidence for Different Mechanisms of Chloroquine Resistance in 2 *Plasmodium* Species That Cause Human Malaria

Takashi Nomura,[1,a] Jane M-R. Carlton,[2] J. Kevin Baird,[5] Hernando A. del Portillo,[6] David J. Fryauff,[3] Dharmendar Rathore,[1] David A. Fidock,[1,a] Xin-zhuan Su,[1] William E. Collins,[4] Thomas F. McCutchan,[1] John C. Wootton,[2] and Thomas E. Wellems[1]

[1]Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, and [2]Computational Biology Branch, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, and [3]Malaria Program, Naval Medical Research Center, Silver Spring, Maryland; [4]Centers for Disease Control and Prevention, Chamblee, Georgia; [5]US Naval Medical Research Unit No. 2, Jakarta, Indonesia; [6]Departamento de Parasitologia, Universidade de São Paulo, São Paulo, Brazil

Chloroquine (CQ)–resistant *Plasmodium vivax* malaria was first reported 12 years ago, nearly 30 years after the recognition of CQ-resistant *P. falciparum.* Loss of CQ efficacy now poses a severe problem for the prevention and treatment of both diseases. Mutations in a digestive vacuole protein encoded by a 13-exon gene, *pfcrt,* were shown recently to have a central role in the CQ resistance (CQR) of *P. falciparum.* Whether mutations in *pfcrt* orthologues of other *Plasmodium* species are involved in CQR remains an open question. This report describes *pfcrt* homologues from *P. vivax, P. knowlesi, P. berghei,* and *Dictyostelium discoideum.* Synteny between the *P. falciparum* and *P. vivax* genes is demonstrated. However, a survey of patient isolates and monkey-adapted lines has shown no association between in vivo CQR and codon mutations in the *P. vivax* gene. This is evidence that the molecular events underlying *P. vivax* CQR differ from those in *P. falciparum.*

In malaria campaigns, the morbidity and mortality of *Plasmodium falciparum* malaria in Africa is often emphasized, whereas the global impact of the less deadly malaria caused by *P. vivax* is overshadowed. Although *P. vivax* malaria rarely is fatal, it is, nevertheless, a debilitating disease with tremendous impact on the quality of life and economic productivity. Estimates of the annual number of *P. vivax* cases range from 75 to 90 million, with most occurring outside Africa. Chloroquine (CQ) has been the drug of choice for eliminating *P. vivax* blood stages, but resistance has been an increasing problem since it was first reported in Papua New Guinea in 1989 [1] and Indonesia in 1991 [2, 3]. Field trials in Papua (Indonesian New Guinea, formerly known as Irian Jaya) showed recrudescence in 22% of patients with *P. vivax* after treatment with CQ [4]. CQ resistance (CQR) also was found in 14% of *P. vivax* infections in an island population off the coast of northwestern Sumatra [5]. The geographic distribution of CQ-resistant *P. vivax* has extended more recently to India [6, 7], Myanmar [8, 9], and, in the Western Hemisphere, to Guyana [10] and Brazil [11].

Despite comparable use of CQ against *P. vivax* and *P. falciparum* malaria throughout the second half of the 20th century, the emergence of CQR in *P. vivax* 3 decades after that of *P. falciparum* [12, 13] and the unique recurrence profile of CQ-resistant *P. vivax,* compared with that of CQ-resistant *P. falciparum* in vivo [14], suggest the possibility of different resistance mechanisms between the 2 species. A gene *(pfcrt)* with a central role in the CQR of *P. falciparum* has been described recently [15]. Point mutations in the encoded PfCRT protein were found to associate completely with the in vitro laboratory measurements of CQR in *P. falciparum* lines from Asia, Africa, and South America. The importance of these mutations was reinforced further by genetic transformation results and by a West African study that showed that PfCRT K76T and certain other accompanying amino acid changes were selected in 100% of patients in whom treatment failed, compared with the baseline prevalence of ~40% in the overall population [16]. PfCRT is a transmembrane protein that localizes to the parasite digestive vacuole, the site of CQ action [17]. Increased acidification of this vacuole associates with PfCRT mutations and

Received 27 November 2000; revised 20 February 2001; electronically published 27 April 2001.

Informed written consent was obtained from patients and/or their guardians under protocols approved by institutional review boards of the US Navy and the Indonesian Ministry of Health, National Institutes of Health Research and Development (protocols DOD 30820 and 30836).

Malaria parasites from animals were produced and treated under protocols (1048COLMONB, 1049COLMONB, and 1127COLCHIX), as reviewed and approved by the Centers for Disease Control and Prevention Animal Care and Use Committee, in accordance with procedures described in the 1986 US Public Health Service Policy.

Financial support: US Naval Medical Research and Development Command (work units 6.2/E/EEX/2405 and 6.3E/HEX/2406); US Veteran's Administration (Mechanisms of Resistance to Chloroquine in *Plasmodium vivax*) 98-FRS-0273.

[a] Present affiliations: Institute for Frontier Medical Sciences, Kyoto University, Sakyo-ku, Kyoto, Japan (T.N.); Albert Einstein College of Medicine, Bronx, New York (D.A.F.).

Reprints or correspondence: Dr. T. E. Wellems, Bldg. 4, Rm. 126, National Institutes of Health Campus, Bethesda, MD 20892-0425 (tew@helix.nih.gov).

The Journal of Infectious Diseases 2001; 183:1653–61

0022-1899/2001/18311-0014$02.00

CQR [15]. Therefore, the question arises as to whether mutations in an orthologue of *pfcrt* may be similarly responsible for CQR in *P. vivax*.

The genus *Plasmodium* comprises deeply branched clades of what are considered to be 3 major evolutionary groups. Representative members of these groups include the following: *P. falciparum,* the closely related ape parasite *P. reichenowi,* and avian malaria parasites, such as *P. gallinaceum; P. vivax* and monkey malarias, such as *P. knowlesi;* and rodent malaria parasites, such as *P. berghei, P. chabaudi,* and *P. yoelii* [18]. Major differences in host specificity and disease manifestations occur among species of these groups, as do dramatic variations in genome composition and codon usage [19].

Here we describe *pfcrt* orthologues from *P. vivax, P. knowlesi,* and *P. berghei* and a *pfcrt* homologue from the slime mold *Dictyostelium discoideum,* and present evidence for different mechanisms of CQR in *P. falciparum* and *P. vivax*.

Materials and Methods

Preparation of polymerase chain reaction (PCR) templates from blood samples. *P. vivax*–infected monkey red blood cells were lysed and washed with Tris-EDTA buffer (10 m*M* Tris-HCl and 1 m*M* disodium EDTA [pH 7.6]) and RCLB solution (1 *M* sucrose, 10 m*M* Tris-HCl [pH 7.6], 5 m*M* $MgCl_2$, and 1% [vol/vol] Triton X100 in H_2O). Pellets were resuspended in DSP (20 m*M* Tris-HCl [pH 7.6], 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 1 g/L proteinase K, and 0.5% Tween 20) and were incubated at 56°C for 2 h and then at 90°C for 10 min. Two microliters of this solution was used for each PCR assay. Dried blood samples from transmigrated Javanese patients infected with *P. vivax* were sectioned into 3-mm^2 pieces and were fixed with methanol for 15 min at room temperature. After drying, the pieces were soaked in 50 μL of H_2O, and 5 μL of the recovered solution was used for each PCR reaction.

Isolation of pfcrt *homologues from* D. discoideum, P. knowlesi, P. vivax, *and* P. berghei. PCR amplifications were performed using standard reagents and 50 ng of genomic DNA or 0.1 μL of heat-denatured DNA from cDNA or genomic DNA libraries as template. Extension temperatures were 64°C for *P. knowlesi* DNA and 72°C for *P. vivax* DNA. The *D. discoideum* clone *ssa662* (GenBank accession nos. AU076370 and C83926) was identified by the basic local alignment search tool (BLAST) screening of National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/BLAST) and was obtained from Hideko Urushihara (Institute of Biological Sciences, University of Tsukuba, Japan) for sequencing.

PCR amplifications with 4 different primer pairs were used to identify the *P. knowlesi* orthologue *pkcg10.* PCR primer pairs BF92 (5′-GCT TTT CAA ACA TGA CAA GGG-3′) and BB103C (5′-TTG CTG GAC CTT GTA TAC AAC TAA C-3′), which were designed from exons 5 and 10 of *pfcrt*, respectively, amplified a 0.5-kb cDNA fragment encoding a central part of *pkcg10* from an erythrocytic-stage *P. knowlesi* λgt11 cDNA library [20]. Primer KB1 (5′-CCC AAT CCA CAG TTC TCC ACA ATA G-3′), which was designed from the sequence of the first product, and primer AF12B (5′-GGT AAC TAT AGT TTT GTA ACA TCC G-3′), which was designed from *pfcrt* exon 2, amplified a further 0.6-kb cDNA fragment from *pkcg10*. Primer K4B7 (5′-GAA TGT TTC CAG TGG TTC TTG TGA GG-3′), which was designed from the aforementioned 0.6-kb cDNA fragment, and the vector-specific primer GT11F3 (5′-TAT GGG GAT TGG TGG CGA CGA CTC C-3′) amplified the 5′ untranslated sequence of the *P. knowlesi* orthologue. Primer KAF22 (5′-GCG ACG ATT GTG AAG GAG CAT GG-3′), which was designed from the first (0.5 kb) fragment, and the vector-specific primer GT11F3 (5′-TAT GGG GAT TGG TGG CGA CGA CTC C-3′) amplified the 3′ untranslated region (UTR) of *pkcg10.*

PCR amplifications and genomic library screening were used to identify the *P. vivax* orthologue *pvcg10.* Primers KF4 (5′-CCC TTC TCG GAA ATC GGA ACG-3′) and KB2 (5′-CAA TCG TCG CAC ATC TTG G-3′) were designed from the *pkcg10* sequence and were used to amplify a 0.3-kb genomic DNA fragment spanning exons 8 and 9 from DNA of *P. vivax* Salvador I (Sal I) line. This fragment was used to probe and to identify a 6-kb *Hin*dIII insert from a *P. vivax* Salvador I genomic DNA library [21]. The 6-kb DNA insert spanned exons 6–14 and the 3′ UTR of the *P. vivax* orthologue. Primers K6F (5′-GAT GAA CGT TAC CGG GAG TTG G-3′) and K6B45 (5′-GCA ATG ATA ACT GAG CAG GCG TC-3′) were designed from the *P. knowlesi pkcg10* sequence and were used to amplify genomic sequence containing exons 1–3 of *pvcg10.* Primers V2F20 (5′-GCA GGA GAG ACA CCG AAA C-3′) and VK2B4 (5′-ATC GGA AGC ATC AGG CAG GAG G-3′) were used to amplify a fragment containing exons 3–6 of *pvcg10*. Primer VB2 (5′-TGC GTC TCG GAG GTT ACA AAG C-3′) and the M13 forward primer M13F1 (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′) were used to amplify a fragment extending into the 5′ UTR of *pvcg10*.

Expressed sequence tag (EST) and genome survey sequence deposits corresponding to *P. berghei* orthologue *pbcg10* were identified by BLAST screening of the University of Florida *Plasmodium berghei* Gene Sequence Tag Project via the NCBI Malaria Genetics and Genomics World Wide Web pages (http://www.ncbi.nlm.nih.gov/Malaria/). Clones UFL_244PbC09, UFL_056PbB12, and UFL_053PbC06 were obtained and sequenced to obtain the full open-reading frame (ORF) of *pbcg10.* Sequence data from this work are deposited in the DNA DataBank of Japan, European Molecular Biology Laboratory, and GenBank databases under accession numbers AF314645–AF314649 and AF317500.

Identification of P. vivax *orthologues* pvcg9, pvcg1, *and* pvcg4. The 6-kb *P. vivax* genomic DNA clone that contained exons 6–14 and the 3′ UTR of *pvcg10* was sequenced further, and ORFs of *pvcg9* and *pvcg1* were identified. Oligonucleotide primers 5′-TGG ACA ACC ACT CCC TGG ACT CTA CCT-3′ and 5′-GCT TTT GCT CAT TTT TCT GCC-3′, which originally were designed to amplify isoleucine tRNA synthase from *P. vivax,* amplified a 1.1-kb fragment from *P. vivax* North Korea genomic DNA. The fragment was cloned into vector pCR2.1 (Invitrogen), was sequenced from both ends, using primer sites in the vector, and was found by database searches to have homology to *pfcg4.* Probes for *pvcg1* were amplified by use of primer pairs VCG1F (5′-CCA TAC ACC AAA CTG GAC TCG G-3′) and VCG1B (5′-GCT AAA CAC GCA ACT GCT GAT GAG-3′), and probes for *pvcg4* were amplified by use of primer pairs 1474 (5′-ATG TCC GTG CTC GGA

ATC GAC ATC GGA AAT GAC-3′) and 1475 (5′-CTC TAC CTG GTA GCC CAG GTA GTT ATG CTC-3′).

Pulsed-field gel electrophoresis (PFGE). Yeast harboring yeast artificial chromosome (YAC) A8 [22] was incubated in a sorbitol solution (0.1 *M* disodium EDTA, 0.1 *M* Tris–HCl [pH 8.0], and 0.9 *M* sorbitol in H_2O) with 28 m*M* β-mercaptoethanol and 1000 U/mL of Lyticase (Sigma). The resulting spheroplasts were embedded in 0.5% (wt/vol) agarose (SeaPlaque; FMC BioProducts), and the blocks were treated with EPS (1% wt/vol sodium N-lauroylsarcosine and 2 mg/mL of proteinase K in 0.5 *M* disodium EDTA) at 37°C for 48 h. Chromosomes were separated for 48 h by PFGE (14°C, 180 V, 0.2 A, switching times ramped from 10 s to 40 s) in 0.8% agarose gel with 0.5× TBE buffer (10× TBE buffer is composed of 108 g of Tris base, 54 g of boric acid, and 8.35 g of disodium EDTA per liter of H_2O) and were blotted onto nylon membrane.

Papua case study design. Javanese transmigrants residing in the hyperendemic Bonggo District of coastal Papua, self-reported to a research team–operated clinic with complaints of symptoms consistent with malaria. All diagnoses were made by examination of Giemsa-stained blood films by expert microscopists, using standard light microscopy. Patient assessment, treatment, and follow-up were carried out as described elsewhere [23]. *P. vivax* parasites were classified as CQ sensitive or resistant, according to the results of a standard 28-day in vivo drug test, which takes into account whole blood levels of CQ and desethylchloroquine at the time of recurrence of parasitemia [14].

Results

Conserved features of a novel family of transporter protein homologues in P. vivax, P. knowlesi, P. berghei, *and* D. discoideum. Investigation of homologues from other organisms can reveal regions of the *pfcrt* gene and its PfCRT product that have been maintained in evolution by structural and functional constraints. Therefore, we used in silico sequence analysis and targeted PCR screening with codon-adjusted primers to identify *pfcrt* orthologues in representatives of 3 deeply branched evolutionary lineages of the genus *Plasmodium* and a more distant homologue from *D. discoideum.* Sequence database searches using BLAST programs [24, 25] gave a significant match between part of the *P. falciparum pfcrt* coding sequence and 2 partial cDNA sequences from the *D. discoideum* cDNA project (GenBank accession nos. AU07268 and C83926) [26]. Inspection of the amino acid sequence encoded by these 2 ESTs indicated potential similarities to the structure of the PfCRT C-terminal region. Therefore, the full-length coding region from one of the corresponding *D. discoideum* cDNA clones, *ssa662,* was determined. The predicted SSA662 amino acid sequence from this coding region demonstrated homology with PfCRT along its entire length (figure 1).

To identify *pfcrt* homologues in representative species of the 3 evolutionary branches of *Plasmodium,* we first used codon-adjusted primers from the *pfcrt*/*ssa662* alignment to amplify fragments from a *P. knowlesi* blood-stage cDNA library. Analysis of these cDNA fragments identified an ORF encoding a *pfcrt* homologue, which we termed *pkcg10* (figure 1). Conserved regions of this homologue, in addition to *pfcrt,* then were employed to obtain a complete genomic sequence of a *pfcrt* homologue of *P. vivax.* Initial amplifications of genomic DNA produced probes that were used to screen a *P. vivax* genomic DNA library. Analysis of these fragments identified the full *P. vivax* coding sequence of a *pfcrt* homologue, which we term *pvcg10.* Partial sequence of a *pfcrt* homologue of the rodent malaria parasite *P. berghei, pbcg10,* was identified through a search of the University of Florida Gene Sequence Tag Project databases [28]. Representative cDNA and genomic clones were obtained and sequenced in their entirety, to yield the complete *pbcg10* coding sequence (figure 1).

Comparative alignments of the amino acid sequences translated from *pfcrt, pvcg10, pkcg10, pbcg10,* and *D. discoideum* clone *ssa622* show striking conservation in overall composition and structural features (figure 1). Consensus prediction indicates that these proteins have 10 transmembrane segments, which are conserved in spacing and orientation. The transmembrane helix-end motifs are consistent with a model in which the N- and C-termini of the proteins are located on the cytoplasmic side of the membrane. Motif searches showed no evidence for the presence of a typical signal sequence or other recognizable feature, such as an ATP-binding motif. These properties suggest that PfCRT, PvCG10, PkCG10, PbCG10, and SSA662 are members of a previously undescribed family of transporter proteins.

Striking conservation in the positions of intron-exon junctions inferred from the genomic sequences of *pfcrt* and *pvcg10* also was apparent. Figure 2 shows a schematic diagram of the arrangement of the coding regions in the exons of *pfcrt* and *pvcg10.* The *pvcg10* coding sequences extend across 14 exons, which range from 45 to 266 bp and are interrupted by 13 introns ranging from 114 to 491 bp. The splice sites of 12 of 13 introns in *pvcg10* are found at positions identical to those of the 12 introns in *pfcrt.* The additional intron in *pvcg10* has no counterpart in *pfcrt* and splits the reading frame that corresponds to *pfcrt* exon 3. The compositional properties of the introns differ dramatically between *pfcrt* and *pvcg10,* which is consistent with the genome-wide difference among the G+C contents of *P. falciparum* (~18% G+C) and *P. vivax* (~45% G+C).

Synteny between P. falciparum *and* P. vivax pfcrt *orthologues and nearby genes.* Comparative sequence analysis demonstrated a close relationship between *pfcrt* and *pvcg10;* however, further evidence that these genes are orthologues was desirable. Therefore, syntenic relationships were investigated between the genes neighboring *pfcrt* and *pvcg10* in the *P. falciparum* and *P. vivax* chromosomes. First, partial sequencing of the 6-kb *P. vivax* genomic library clone, from which exons 6–14 of *pvcg10* were identified, was used to identify 2 ORFs 4-kb downstream of *pvcg10,* in the same position as the *P. falciparum pfcg9* and *pfcg1* genes relative to *pfcrt* (figure 2). Translation of the *pvcg9* and *pvcg1* ORFs yielded amino acid sequences with 32% and

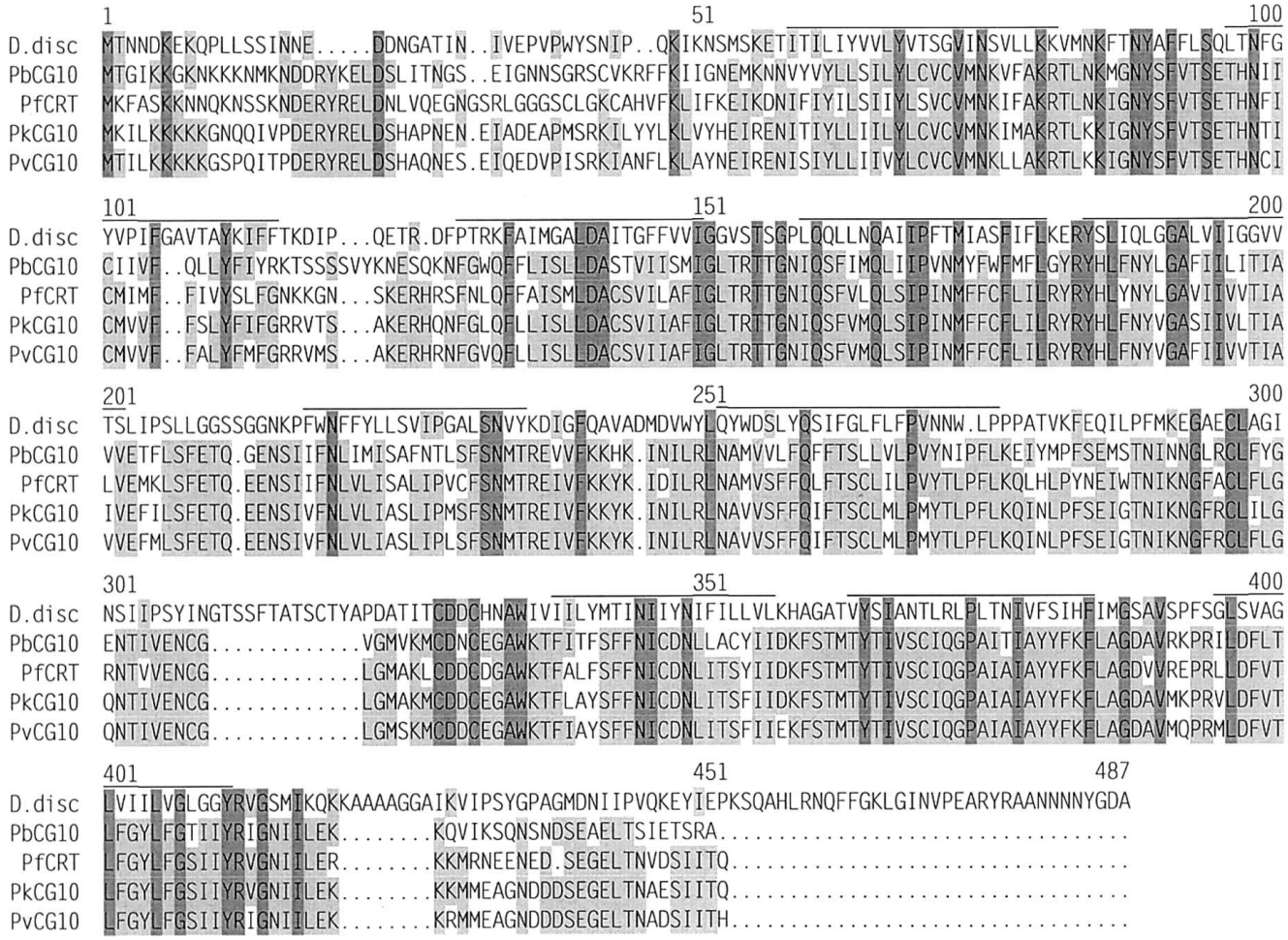

Figure 1. Comparison of the predicted sequences for PfCRT (clone HB3) and its homologues PvCG10 (Salvador I), PkCG10, PbCG10, and *Dictyostelium discoideum* (D.disc) SSA662. Matching amino acids across all species are shaded darkly, whereas matching amino acids in ⩾3 species are shaded lightly. Lines over sequences indicate putative transmembrane segments predicted by TMHMM [27].

39% amino acid identity to the predicted PfCG9 and PfCG1 sequences, respectively (data not shown). A third partial sequence of a *P. vivax* gene, *pvcg4,* also was recognized in a serendipitous product in separate PCR amplifications of genomic DNA. The amino acid translation from *pvcg4* showed 75% identity with the predicted sequence from *pfcg4* over 105 residues (data not shown). This relationship provided another probe to investigate synteny, since *pfcg4* is located ~5 kb from *pfcrt* on *P. falciparum* chromosome 7 [15].

To confirm synteny in the organization of these *P. vivax* and *P. falciparum* genes, a YAC library containing large inserts of *P. vivax* DNA [22] was screened with PCR primer pairs specific for *pvcg10.* A clone identified by this screen, YAC A8, was found to harbor a 350-kb insert that hybridized to the separate *pvcg10, pvcg4,* and *pvcg1* probes (figure 3). These results established the presence of syntenic relationships upstream and downstream of *pfcrt* and *pvcg10.* Given this, their close homology, and the almost identical intron-exon organization of these genes, we conclude that *pfcrt* and *pvcg10* are true orthologues within this family of transporter genes.

PvCG10 mutations are not associated with CQR in monkey-adapted P. vivax *isolates.* In contrast to the case for *P. falciparum, P. vivax* cannot be consistently maintained in vitro [29]. Practicable in vitro drug susceptibility assays on cultivated parasite lines are therefore not possible. Instead, the resistance status of the parasite is usually determined from patients' clinical responses or from in vivo responses of adapted lines maintained in monkeys or chimpanzees. Therefore, we studied 14 isolates of *P. vivax* maintained at the Centers for Disease Control and Prevention (CDC) by serial passage in monkeys (table 1).

Of the 14 monkey-adapted strains, 3 were CQ resistant. One strain, AMRU-1, which originally was isolated from an Australian serviceman stationed in Papua and for whom CQ treatment failed [1], was adapted to growth in *Aotus* monkeys [44] and consistently failed to clear at doses ⩽30 mg/kg of CQ administered over 3 days [31]. The second strain, Indonesia

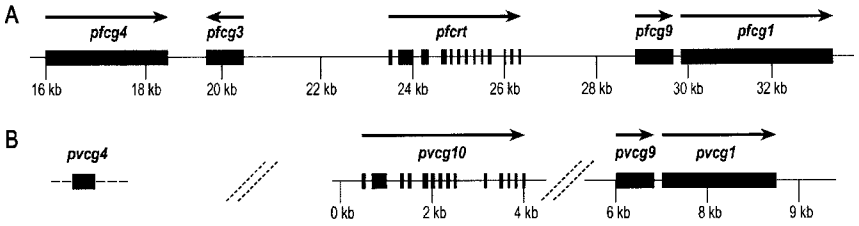

Figure 2. Intron-exon organization of *pfcrt* and *pvcg10* and surrounding genes. *A,* Gene structure of a part of the 38-kb *Plasmodium falciparum* chromosome 7 region (GenBank accession no. AF030694) [15] showing the relative positions of *pfcg4, pfcg3, pfcrt, pfcg9,* and *pfcg1. B,* Gene structure of *Plasmodium vivax* DNA containing orthologues *pvcg10, pvcg9,* and *pvcg1.* Boxes indicate exons, and arrows indicate gene direction. Note that the organization of *pfcrt, pfcg9,* and *pfcg1* is comparable with that of *pvcg10, pfcg9,* and *pvcg1,* which provides evidence that *pvcg10* is an orthologue of *pfcrt*. The probable location of *pvcg4* also is shown. Coding directions indicated by the arrows are assumed and have not been verified by directional mapping.

XIX/CDC *P. vivax,* was isolated from patients in Papua and was resistant to 15 mg/kg of CQ administered over 3 days [30]. The third strain, Indonesia I/CDC, was isolated from a patient on the island of Nias. CQ treatment [3] of the patient was unsuccessful, and the strain was adapted to growth in *Aotus* monkeys, in whom it was found to be resistant to treatment with 15 mg/kg of CQ given as a single dose [32]. The resistant phenotypes of these 3 strains are in direct contrast to the sensitive phenotypes of the 11 strains listed in table 1, which were collected before successful CQ treatment of patients.

The *pvcg10* coding sequences of these isolates were compared. The 14 exons of 2 CQ-resistant (Indonesia XIX and AMRU-1) and 2 CQ-sensitive (Chesson and Salvador I) strains were sequenced in their entirety. The only nonsynonymous point mutations identified were at amino acid positions 34 and 38 (Chesson) and 384 (AMRU-1). Therefore, exons containing codons 34 and 38 were sequenced in the 10 remaining isolates. These exons showed identical *pvcg10* haplotypes (table 2). These results demonstrate a lack of association between codon mutations in *pvcg10* and in vivo CQ susceptibility of the *P. vivax* monkey-adapted lines.

PvCG10 mutations are not associated with CQR in P. vivax *patient isolates from Papua.* To test further our PvCG10 mutation findings, we sequenced *pvcg10* alleles of *P. vivax* obtained from patients who lived in an endemic area and for whom CQ treatment in vivo had failed. Stringent criteria are necessary to make definitive pronouncements of in vivo resistance to therapeutic levels of CQ. These criteria include supervision of the appropriate dose of CQ, monitoring of parasitemia, and the demonstration of parasites in the presence of CQ blood levels that are greater than the minimally effective concentration (100 ng/mL for *P. vivax*) derived from CQ-sensitive strains of this parasite [14]. Using these criteria, 8 *P. vivax* isolates were obtained from Papuan patients with CQ-sensitive or -resistant infections. Four of these infections were classified as clinically resistant, 3 as clinically sensitive, and 1 as probably resistant, because the measured blood level of 85 ng/mL was below the formal cutoff of 100 ng/mL (table 3).

The sequences of all exons of *pvcg10* were determined for 2 resistant (CL002 and CL004), 1 sensitive (CL007), and the 1 probably resistant sample (CL003). No new nonsynonymous mutations were found in these sequences. In light of these results, the re-

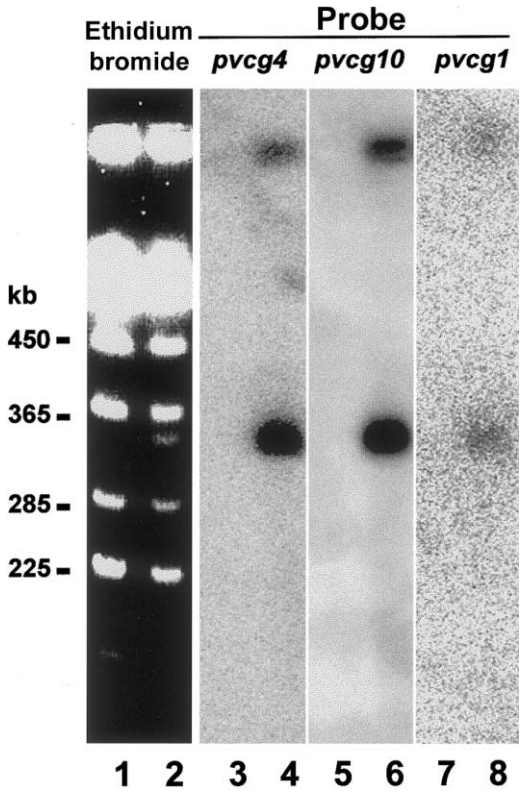

Figure 3. Hybridization of *pvcg4, pvcg10,* and *pvcg1* probes to *Plasmodium vivax* yeast artificial chromosome (YAC) A8 separated by pulsed-field gel electrophoresis. YAC A2 (150 kb) and YAC A8 (350 kb) are loaded on odd and even lanes, respectively. Gel was stained with ethidium bromide *(lanes 1* and *2)* and was blotted onto nylon membrane for probing with *pvcg4 (lanes 3* and *4), pvcg10 (lanes 5* and *6),* and *pvcg1 (lanes 7* and *8).*

Table 1. Origin, isolation, and chloroquine (CQ) response data for 14 lines of monkey-adapted *Plasmodium vivax.*

Line	Country	Isolation and monkey adaptation	In vivo CQ phenotype; reference
Indonesia XIX	Indonesia	Collins et al. [30]	R; Collins et al. [30]
AMRU-1	Papua New Guinea	Obaldia et al. [31]; Rieckmann et al. [1]	R; Obaldia et al. [31]
Indonesia I/CDC	Indonesia	Collins et al. [32]; Schwartz et al. [3]	R; Collins et al. [32]
Chesson	Papua New Guinea	Collins et al. [33]; Ehrman et al. [34]	S; Schmidt [35]
Salvador I	El Salvador	Collins et al. [36]; Contacos et al. [37]	S; Contacos et al. [37]
New Guinea I/CDC	Papua New Guinea	Collins et al. [38]	S; Collins (unpublished)
New Guinea IV/CDC	Papua New Guinea	Collins (unpublished)[a]	S; Collins (unpublished)
Sumatra	Sumatra	Collins (unpublished)[b]	S; Collins (unpublished)
Palo Alto	Vietnam	Collins et al. [38]; Geiman and Meagher [39]	S; Schmidt [35]
North Korea	North Korea	Collins et al. [40]	S; Collins (unpublished)
Thai III	Thailand	Collins et al. [41]	S; Collins (unpublished)
India VII	India	Collins (unpublished)[c]	S; Collins (unpublished)
Brazil I/CDC	Brazil	Nayar et al. [42]	S; Nayar et al. [42]
Mauritania I	Mauritania	Collins et al. [43]	S; Collins (unpublished)

NOTE. CDC, Centers for Disease Control and Prevention; R, resistant, and S, sensitive (as described in the text).
[a] Isolated from an infected patient on return from New Guinea, June 1992.
[b] Isolated from an oil worker in Sumatra. Patient blood was used to infect *Aotus* monkeys, November 1993.
[c] India VII isolated from frozen blood sample from India. Adapted to *Aotus* monkeys, April 1995.

mainder of the samples were sequenced for exons containing codons 34, 38, and 384 (table 2). The results confirm no identifiable association between codon mutations in *pvcg10* and CQ susceptibility. All patient blood samples showed identical *pvcg10* haplotypes except sample CL002, which showed a Leu→Phe change at position 384, which was similar to the AMRU-I monkey-adapted strain. In addition, a silent mutation in codon 343 that codes for Thr distinguished all 8 patient blood samples (ACC) from all 14 monkey-passaged isolates (ACG; data not shown).

Ancestral haplotype of CRT homologues inferred from species of Plasmodium. Is there further evidence that points to the lack of a role for *pvcg10* codon mutations in clinically CQ-resistant *P. vivax?* The identification and sequencing of the complete coding sequence of 3 PfCRT homologues, in conjunction with mutation data from CQ-resistant and -sensitive clones of *P. falciparum* [15], can be used to infer the ancestral *Plasmodium* gene haplotype listed in table 4. All CQ-resistant *P. falciparum* strains from Asia, Africa, and South America analyzed thus far show distinct *pfcrt* alleles that differ from this canonical, sensitive allele by ⩾4 point mutations; amino acid changes encoded by these mutations consistently include K76T and A220S. These mutant alleles in CQ-resistant strains of *P. falciparum* thus show different instances of deviations from the haplotype, which is indicative of strong selective pressure from CQ at this locus. By contrast, the CQ-sensitive and -resistant strains of *P. vivax* show little deviation from the inferred ancestral CRT haplotype. This difference in the mutation of orthologous genes from 2 different species is supportive of a different genetic mechanism of CQR in *P. vivax.*

Discussion

Despite comparable selection pressure from drug use, CQR in *P. vivax* has been reported only since 1989, whereas CQR in *P. falciparum* was evident in the late 1950s from separate foci in South America and Southeast Asia [46]. This history may reflect basic differences in the genetic determinants and molecular mechanisms of CQR in *P. falciparum* and *P. vivax,* which is a hypothesis supported by the results described in this

Table 2. PvCG10 mutations and in vivo chloroquine (CQ) response phenotypes of *Plasmodium vivax* lines from monkey and human infections.

Line or sample	In vivo phenotype	Amino acid position codon 34	38	384
Monkey adapted				
Indonesia XIX	R	Q	P	L
AMRU-1	R	Q	P	**F**
Indonesia I	R	Q	P	—
Chesson	S	**H**	**L**	L
Salvador I	S	Q	P	L
New Guinea I/CDC	S	Q	P	—
New Guinea	S	Q	P	—
Sumatra	S	Q	P	—
Palo Alto	S	Q	P	—
Thai III	S	Q	P	—
India VII	S	Q	P	—
North Korea	S	Q	P	—
Brazil I/CDC	S	Q	P	—
Mauritania I	S	Q	P	—
Patient blood samples (Papua)				
CL002	R	Q	P	**F**
CL004	R	Q	P	L
CL026	R	Q	P	L
CL029	R	Q	P	L
CL003	PR	Q	P	L
CL007	S	Q	P	L
CL001	S	Q	P	L
CL020	S	Q	P	L

NOTE. Underlined isolates were sequenced for all exons of *pvcg10*. Residues and bases in boldface represent identified polymorphic sites. CDC, Centers for Disease Control and Prevention; R, resistant to CQ in vivo; S, sensitive to CQ; PR, probably resistant to CQ (see table 3).

Table 3. Eight-day in vivo chloroquine (CQ) susceptibility results for 8 Javanese transmigrants living in Papua presenting with *Plasmodium vivax* malaria.

Sample	Parasites/μL at day 0[a]	Recurrence[b] (day after CQ therapy[c])	Parasites/μL on day of recurrence	CQ + DCQ blood level, ng/mL[d]	In vivo phenotype
CL002	4000	Yes (7)	360	185	R
CL004	4720	Yes (14)	560	115	R
CL026	5600	Yes (14)	200	190	R
CL029	2120	Yes (18)	240	145	R
CL003	5560	Yes (28)	280	85	PR[e]
CL001	560	No	—	—	S
CL007	2880	No	—	—	S
CL020	600	No	—	—	S

NOTE. DCQ, desethylchloroquine; PR, probably resistant to CQ; R, resistant to CQ; S, sensitive to CQ.

[a] Calculated as 40 × (average no. of parasites per 200 leukocytes), assuming 8000 leukocytes/μL.

[b] Presence of parasites that may be due to relapse, recrudescence, or reinfection.

[c] Patients received standard CQ therapy of 25 mg base/kg body weight over 3 days (10 + 10 + 5 mg/kg doses at 24-h intervals).

[d] Levels of CQ and its major metabolite, DCQ, were determined by high-performance liquid chromatography [45] from samples obtained the day recurrences were identified.

[e] Classified as PR because the measured CQ blood level of 85 ng/mL was below the formal cutoff of 100 ng/mL.

report. Although codon mutations in the *pfcrt* gene are central to the CQR phenotype of *P. falciparum,* no codon mutations in the *pvcg10* gene could be associated with CQR in *P. vivax.* These findings do not, of course, rule out the possibility of expression level adjustments or other changes in the *pvcg10* product having a potential role in CQ-resistant *P. vivax.*

The structure and organization of the *pfcrt* orthologues in *P. falciparum* and *P. vivax* described here are highly informative, as these 2 species are phylogenetically distant and are responsible for most malaria worldwide [47, 48]. The orthologues have a highly interrupted gene structure, with splice junctions at 12 identical positions in the coding frame. The splice junctions differ only by the presence in *pvcg10* of an additional junction between exons 3 and 4, which joins 2 separate exons corresponding to exon 3 of the *P. falciparum* gene. Notwithstanding this conserved intron-exon structure and the high levels of identity and similarity in the encoded amino acid sequences, *pfcrt* and *pvcg10* differ tremendously in codon usage and A/T content. These observations attest to a remarkable maintenance of gene structure and conservation of gene function, despite the dramatic differences in nucleotide content that have arisen since the evolutionary divergence of *P. falciparum* and *P. vivax.*

The concept of different molecular mechanisms underlying a common drug resistance phenotype is not an unusual scenario among microorganisms. In bacteria, for example, despite the fact that target similarities enable certain antimicrobial drugs to act in a fairly uniform fashion regardless of the species, the mechanisms of resistance employed by different species of bacteria to a single drug vary widely [49]. *Plasmodium* species exhibit variations in the length of time for drug resistance to develop [50], in physiologic versus genetic resistance [51], and in the genetic predisposition toward development of resistance [52]. Some of these variations may reflect different features in *Plasmodium* biology that allow for 1 mechanism of resistance to be favored in 1 species but not in another. Of interest, the mechanism of resistance to the antifolate pyrimethamine appears to be similar among most species studied thus far: resistance in *P. falciparum* and in the rodent malarias *P. berghei, P. chabaudi,* and *P. yoelii* [53] and, more recently, in *P. vivax* [54] involves point mutations in the dihydrofolate reductase drug target. Analysis of the *P. vivax cg10* gene presented here is evidence for a unique molecular mechanism of CQR in this important species. Further progress in the development of a continuous culture system and in genetic analysis of *P. vivax* will be required to support further investigations of the CQR mechanism in this species.

Identification of *pfcrt* homologues in *P. falciparum, P. vivax,* and *Plasmodium* species used as model systems for malaria (primate and rodent) should aid investigations into the natural function of these genes. The distantly related *ssa662* gene of *D. dictyostelium* also may provide useful insights into the *pfcrt* homologues, although the value of such insights would necessarily be based on the manipulation of shared functional properties in this organism. In turn, experiments directed to the natural role of the *pfcrt* homologues can be expected to help clarify the function of *pfcrt* itself.

Table 4. Ancestral haplotype of PfCRT inferred from polymorphisms in *Plasmodium* species.

	Amino acid position, PfCRT numbering									
Species	72	74	75	76	97	220	271	326	356	371
P. falciparum,[a] CQ sensitive (canonical form)	C	M	N	K	H	A	Q	N	I	R
P. falciparum,[a] CQ resistant (Old and New World forms)	**C/S**	**I/M**	**E/N**	**T**	**H/Q**	S	E/Q	**S/D/N**	**T/L/I**	**I/R/T**
P. vivax, CQ sensitive and CQ resistant	C	M	N	K	H	S	Q	N	I	**M**
P. knowlesi, CQ sensitive	C	M	N	K	H	S	Q	N	I	**M**
P. berghei, CQ sensitive	C	M	N	K	H	A	E	N	I	R
Inferred ancestral haplotype	C	M	N	K	H	A/S	E/Q	N	I	R

NOTE. Residues marked in boldface differ from the inferred ancestral haplotype. CQ, chloroquine.

[a] From Fidock et al. [15].

Acknowledgments

We are grateful to the *Dictyostelium* cDNA Project in Japan for receipt of clone *ssa662*. We thank Jingtao You and Jianbing Mu (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and Michelle Fluegge (College of Veterinary Medicine, University of Florida, Gainesville) for technical support; Brenda Rae Marshall for editorial assistance; and the National Institute of Health Research, Ministry of Health, the Republic of Indonesia for assistance and guidance in the conduct of the studies in Papua.

References

1. Rieckmann KH, Davis DR, Hutton DC. *Plasmodium vivax* resistant to chloroquine? Lancet **1989**; 2:1183–4.
2. Baird JK, Basri H, Purnomo, et al. Resistance to chloroquine by *Plasmodium vivax* in Irian Jaya, Indonesia. Am J Trop Med Hyg **1991**; 44:547–52.
3. Schwartz IK, Lackritz EM, Patchen LC. Chloroquine-resistant *Plasmodium vivax* from Indonesia [letter]. N Engl J Med **1991**; 324:927.
4. Murphy GS, Basri H, Purnomo, et al. Vivax malaria resistant to treatment and prophylaxis with chloroquine. Lancet **1993**; 341:96–100.
5. Baird JK, Sustriayu Nalim MF, Basri H, et al. Survey of resistance to chloroquine by *Plasmodium vivax* in Indonesia. Trans R Soc Trop Med Hyg **1996**; 90:409–11.
6. Garg M, Gopinathan N, Bodhe P, Kshirsagar NA. Vivax malaria resistant to chloroquine: case reports from Bombay. Trans R Soc Trop Med Hyg **1995**; 89:656–7.
7. Dua VK, Kar PK, Sharma VP. Chloroquine resistant *Plasmodium vivax* malaria in India. Trop Med Int Health **1996**; 1:816–9.
8. Myat PK, Myint O, Myint L, Thaw Z, Kyin HA, Nwe NY. Emergence of chloroquine-resistant *Plasmodium vivax* in Myanmar (Burma) [letter]. Trans R Soc Trop Med Hyg **1993**; 87:687.
9. Marlar T, Myat PK, Aye YS, Khaing KG, Ma S, Myint O. Development of resistance to chloroquine by *Plasmodium vivax* in Myanmar. Trans R Soc Trop Med Hyg **1995**; 89:307–8.
10. Phillips EJ, Keystone JS, Kain KC. Failure of combined chloroquine and high-dose primaquine therapy for *Plasmodium vivax* malaria acquired in Guyana, South America. Clin Infect Dis **1996**; 23:1171–3.
11. Alecrim MG, Alecrim W, Macedo V. *Plasmodium vivax* resistance to chloroquine (R2) and mefloquine (R3) in Brazilian Amazon region. Rev Soc Bras Med Trop **1999**; 32:67–8.
12. Young MD, Moore DV. Chloroquine resistance in *Plasmodium falciparum.* Am J Trop Med Hyg **1961**; 10:317–20.
13. Harinasuta T, Migasen S, Bunnag D. Chloroquine resistance in *Plasmodium falciparum* in Thailand. UNESCO First Regional Symposium on Scientific Knowledge of Tropical Parasites. Singapore: Singapore University Press, **1962**:148–53.
14. Baird JK, Leksana B, Masbar S, et al. Diagnosis of resistance to chloroquine by *Plasmodium vivax:* timing of recurrence and whole blood chloroquine levels. Am J Trop Med Hyg **1997**; 56:621–6.
15. Fidock DA, Nomura T, Talley AK, et al. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Mol Cell **2000**; 6:861–71.
16. Djimde A, Doumbo OK, Cortese JF, et al. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med **2001**; 344:257–63.
17. Goldberg DE, Slater AF, Cerami A, Henderson GB. Hemoglobin degradation in the malaria parasite *Plasmodium falciparum:* an ordered process in a unique organelle. Proc Natl Acad Sci USA **1990**; 87:2931–5.
18. Waters AP, Higgins DG, McCutchan TF. *Plasmodium falciparum* appears to have arisen as a result of lateral transfer between avian and human hosts. Proc Natl Acad Sci USA **1991**; 88:3140–4.
19. McCutchan TF, Dame JB, Miller LH, Barnwell J. Evolutionary relatedness of *Plasmodium* species as determined by the structure of DNA. Science **1984**; 225:808–11.
20. Hudson DE, Wellems TE, Miller LH. Molecular basis for mutation in a surface protein expressed by malaria parasites. J Mol Biol **1988**; 203:707–14.
21. Gibson HL, Tucker JE, Kaslow DC, et al. Structure and expression of the gene for Pv200, a major blood-stage surface antigen of *Plasmodium vivax.* Mol Biochem Parasitol **1992**; 50:325–33.
22. Camargo AA, Fischer K, Lanzer M, del Portillo HA. Construction and characterization of a *Plasmodium vivax* genomic library in yeast artificial chromosomes. Genomics **1997**; 42:467–73.
23. Baird JK, Wiady I, Fryauff DJ, et al. In vivo resistance to chloroquine by *Plasmodium vivax* and *Plasmodium falciparum* at Nabire, Irian Jaya, Indonesia. Am J Trop Med Hyg **1997**; 56:627–31.
24. Altschul SF, Boguski MS, Gish W, Wootton JC. Issues in searching molecular sequence databases. Nat Genet **1994**; 6:119–29.
25. Altschul SF, Madden TL, Schaffer AA, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **1997**; 25:3389–402.
26. Morio T, Urushihara H, Saito T, et al. The *Dictyostelium* developmental cDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. DNA Res **1998**; 5:335–40.
27. Sonnhammer EL, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. ISMB **1998**; 6:175–82.
28. Carlton JM, Dame JB. The *Plasmodium vivax* and *P. berghei* gene sequence tag projects. Parasitol Today **2000**; 16:409.
29. Golenda CF, Li J, Rosenberg R. Continuous in vitro propagation of the malaria parasite *Plasmodium vivax*. Proc Natl Acad Sci USA **1997**; 94:6786–91.
30. Collins WE, Sullivan JS, Fryauff DJ, et al. Adaptation of a chloroquine-resistant strain of *Plasmodium vivax* from Indonesia to New World monkeys. Am J Trop Med Hyg **2000**; 62:491–5.
31. Obaldia N3, Rossan RN, Cooper RD, et al. WR 238605, chloroquine, and their combinations as blood schizonticides against a chloroquine-resistant strain of *Plasmodium vivax* in Aotus monkeys. Am J Trop Med Hyg **1997**; 56: 508–10.
32. Collins WE, Schwartz IK, Skinner JC, Morris C, Filipski VK. The susceptibility of the Indonesian I/CDC strain of *Plasmodium vivax* to chloroquine. J Parasitol **1992**; 78:344–9.
33. Collins WE, Warren M, Contacos PG, Skinner JC, Richardson BB, Kearse TS. The Chesson strain of *Plasmodium vivax* in *Aotus* monkeys and anopheline mosquitoes. J Parasitol **1980**; 66:488–97.
34. Ehrman FCJ, Ellis JM, Young MD. *Plasmodium vivax* Chesson strain [letter]. Science **1945**; 101:377.
35. Schmidt LH. *Plasmodium falciparum* and *Plasmodium vivax* infections in the owl monkey *(Aotus trivirgatus).* II. Responses to chloroquine, quinine, and pyrimethamine. Am J Trop Med Hyg **1978**; 27:703–17.
36. Collins WE, Contacos PG, Krotoski WA, Howard WA. Transmission of four Central American strains of *Plasmodium vivax* from monkey to man. J Parasitol **1972**; 58:332–5.
37. Contacos PG, Collins WE, Jeffery GM, Krotoski WA, Howard WA. Studies on the characterization of *Plasmodium vivax* strains from Central America. Am J Trop Med Hyg **1972**; 21:707–12.
38. Collins WE, Skinner JC, Mehaffey P, Sutton BB. Infection of *Aotus azarae boliviensis* monkeys with different strains of *Plasmodium vivax.* J Parasitol **1985**; 71:239–43.
39. Geiman QM, Meagher MJ. Susceptibility of a New World monkey to *Plasmodium falciparum* from man. Nature **1967**; 215:437–9.
40. Collins WE, Skinner JC, Krotoski WA, et al. Studies on the North Korean strain of *Plasmodium vivax* in *Aotus* monkeys and different anophelines. J Parasitol **1985**; 71:20–7.
41. Collins WE, Sattabongkot J, Wirtz RA, et al. Development of a polymorphic strain of *Plasmodium vivax* in monkeys. J Parasitol **1992**; 78:485–91.
42. Nayar JK, Baker RH, Knight JW, et al. Studies on a primaquine-tolerant

strain of *Plasmodium vivax* from Brazil in Aotus and Saimiri monkeys. J Parasitol **1997**; 83:739–45.

43. Collins WE, Nguyen-Dinh P, Sullivan JS, et al. Adaptation of a strain of *Plasmodium vivax* from Mauritania to New World monkeys and anopheline mosquitoes. J Parasitol **1998**; 84:619–21.
44. Cooper RD. Studies of a chloroquine-resistant strain of *Plasmodium vivax* from Papua New Guinea in *Aotus* and *Anopheles farauti s.l.* J Parasitol **1994**; 80:789–95.
45. Patchen LC, Mount DL, Schwartz IK, Churchill FC. Analysis of filter-paper–absorbed, finger-stick blood samples for chloroquine and its major metabolite using high-performance liquid chromatography with fluorescence detection. J Chromatogr **1983**; 278:81–9.
46. Milhous WK, Kyle DE. Introduction to the modes of action and mechanisms of resistance to antimalarials. In: Sherman IW, ed. Malaria: parasite biology, pathogenesis and protection. Washington DC: American Society of Microbiology Press, **1998**:303–16.
47. Escalante AA, Ayala FJ. Phylogeny of the malarial genus *Plasmodium,* derived from rRNA gene sequences. Proc Natl Acad Sci USA **1994**; 91:11373–7.
48. Escalante AA, Ayala FJ. Evolutionary origin of *Plasmodium* and other Apicomplexa based on rRNA genes. Proc Natl Acad Sci USA **1995**; 92: 5793–7.
49. McManus MC. Mechanisms of bacterial resistance to antimicrobial agents. Am J Health Syst Pharm **1997**; 54:1420–33.
50. Peel SA, Bright P, Yount B, Handy J, Baric RS. A strong association between mefloquine and halofantrine resistance and amplification, overexpression, and mutation in the P-glycoprotein gene homolog *(pfmdr)* of *Plasmodium falciparum* in vitro. Am J Trop Med Hyg **1994**; 51:648–58.
51. Peters W. Drug resistance in *Plasmodium berghei* Vincke and Lips, 1948. I. Chloroquine resistance. Exp Parasitol **1965**; 17:80–9.
52. Rathod PK, McErlean T, Lee PC. Variations in frequencies of drug resistance in *Plasmodium falciparum*. Proc Natl Acad Sci USA **1997**; 94:9389–93.
53. Carlton JMR, Hayton K, Cravo PVL, Walliker D. Of mice and malaria mutants: unravelling the genetics of drug resistance using rodent malaria models. Parasitol Today (in press).
54. de Pecoulas PE, Tahar R, Ouatas T, Mazabraud A, Basco LK. Sequence variations in the *Plasmodium vivax* dihydrofolate reductase–thymidylate synthase gene and their relationship with pyrimethamine resistance. Mol Biochem Parasitol **1998**; 92:265–73.