[Jpn. J. Parasitol., Vol. 45, No. 5, 373–383, October, 1996]

Cytochrome *c* Oxidase III (COIII) Gene of *Plasmodium vivax*: Complete Coding Sequence and Its Expression in the Erythrocytic Stages of the Parasite

PATRICIA LIM KIM CHOOI[1)], YOH-ICHI WATANABE[2,3*)], TAN SIEW KIAN[1)], TAKAHISA FURUTA[2,3)], TAKASHI AOKI[2,4)], SOMEI KOJIMA[2,3)], KIYOSHI KITA[2,3)], YAGYA D. SHARMA[5)] AND MAK JOON WAH[1)]

[1)]*Division of Molecular Pathology, Institute for Medical Research, Jalan Pahang 50588, Kuala Lumpur, Malaysia.*
[2)]*Institute for Medical Research and Japan International Cooperation Agency Research Project on Tropical Diseases, Institute for Medical Research, Jalan Pahang 50588, Kuala Lumpur, Malaysia.*
[3)]*Department of Parasitology, The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, Japan.*
[4)]*Department of Parasitology, Juntendo University, School of Medicine, Hongo, Bunkyo-ku, Tokyo 113, Japan.*
[5)]*Department of Biotechnology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110 029, India.*
**Present Address: Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan.*
(Accepted October 25, 1996)

Abstract

In malaria parasites, there is the 6 kb DNA element which encodes cytochrome *c* oxidase subunit I, subunit III (COIII), apocytochrome *b* and fragmented ribosomal RNAs genes. Previously, we have amplified a partial DNA fragment of the COIII gene of *Plasmodium vivax*, the human malaria parasite, using PCR primers derived from *P. falciparum* sequence (Lim *et al.*, 1995). In this study, we amplified two other DNA fragments of *P. vivax* using PCR primers derived from either *P. falciparum* or *P. vivax* sequences to cover the whole COIII gene region. The possible open reading frame in the determined sequence is 792-nucleotides long. The complete COIII sequences of *P. vivax* and *P. falciparum* are 71% nucleotide and 73% amino acid identical, while the *P. vivax* and *P. yoelii* sequences are 81% nucleotide and 82% amino acid identical. We have detected RT-PCR products using COIII gene specific primers and oligo(dT)– or random-primed cDNA from poly(A)+ RNA from the erythrocytic stage of *P. vivax*. The results suggest that the COIII gene of *P. vivax* is functional in its erythrocytic stages and that the COIII gene transcript has the 3'-poly(A) sequence.

Key words: *Plasmodium vivax*; cytochrome *c* oxidase; 6 kb DNA.

Correspondence: Kiyoshi Kita, kitak@ims.u-tokyo.ac.jp
Patricia Lim Kim Chooi[1], 渡邊洋一[2,3], Tan Siew Kian[1], 古田隆久[2,3], 青木　孝[2,4], 小島莊明[2,3], 北　潔[2,3], Yagya D. Sharma[5], Mak Joon Wah[1]
([1]Division of Molecular Pathology, Institute for Medical Research, [2]JICA マレーシア国熱帯病研究プロジェクト, [3]東京大学医科学研究所寄生虫研究部, [4]順天堂大学医学部寄生虫学教室, [5]Department of Biotechnology, All India Institute of Medical Sciences)

This study was performed as a part of the Institute for Medical Research and Japan International Cooperation Agency Research Project on Tropical Diseases technically managed by Dr. H. Tanaka, chief adviser, and was supported by a Grant-in-Aid for Scientific Research on Priority Areas (Nos. 08281103 and 08281105) from the Ministry of Education, Science, Sports and Culture of Japan for S.K. and K.K., respectively.

Introduction

Energy production of *Plasmodium*, the malaria parasite, in erythrocytic stages is mainly via glycolysis and key enzymes in TCA cycle are absent (Sherman, 1979). However, cytochrome oxidase activity (Scheibel and Pflaum, 1970) and cytochrome components of the electron transport system (Fry and Beelsley, 1991) were detected. Further, the maintenance of a high transmembrane potential of mitochondria (Divo *et al.*, 1985b) suggested the function of mitochondria in energy metabolism and pyrimidine biosynthesis (Gutteridge *et al.*, 1979; Krungkrai, 1995). The antimalarial effects of inhibi-

tors of organelle protein synthesis and the electron transport system also supports this idea (Geary and Jensen, 1983, Divo *et al.*, 1985; Ginsburg *et al.*, 1986).

On the other hand, recent molecular biological studies revealed the presence of two kinds of extrachromosomal DNA in malaria parasite (Feagin, 1994). One is the 35 kilo-base pair (kb) circular DNA which is suggested to be related to plastid DNA (Feagin, 1994). The other is a 6 kb, tandemly repeated element which has three genes homologous to those for respiratory proteins encoded in mitochondrial DNA: cytochrome *c* oxidase subunits I and III (COI and COIII) and apocytochrome *b* (cyt*b*), as well as highly fragmented ribosomal RNAs (rRNA) (Vaidya *et al.*, 1989; Vaidya *et al.*, 1990; Aldritt *et al.*, 1989; Suplick *et al.*, 1990; Joseph *et al.*, 1989; Feagin, 1992; Feagin *et al.*, 1992; Vaidya *et al.*, 1993). The fact that this DNA is co-purified with mitochondrial enzyme activities supported the mitochondrial origin of this DNA (Wilson *et al.*, 1992). The transcription of open reading frames in this DNA as well as fragmented rRNA genes also suggested that the genes in this DNA are functional (Aldritt *et al.*, 1989; Feagin *et al.*, 1992; Joseph *et al.*, 1989; Suplick *et al.*, 1990; Vaidya and Arasu, 1987; Vaidya *et al.*, 1993; Feagin and Drew, 1995; Feagin, 1992). In addition, the 6 kb elements was suggested to be a diagnostic target because of its multiple copy number in the parasite (Joseph *et al.*, 1989; Vaidya and Arasu, 1987; Preiser *et al.*, 1996).

Although the respiratory chain components encoded in the 6 kb DNA have been suggested as possible targets for chemotherapy, only the sequence information of *Plasmodium falciparum* among human malaria parasites is available (Feagin, 1992; Feagin *et al.*, 1992; Vaidya *et al.*, 1993) because of the limitation of the starting material of other human malaria parasites. To overcome the problem, in the previous report, we applied PCR technique to get partial fragment of the 6 kb DNA of the human malaria parasite, *P. vivax*, and reported the partial sequence of COIII gene of the parasite (Lim *et al.*, 1995). In this report, we present the complete coding sequence of COIII gene of *P. vivax* as well as the detection of the transcription of the gene in the erythrocytic stages of the parasite.

Materials and Methods

DNA

Total DNA of *P. vivax* was isolated from a Malaysian student with the infection acquired in India and the single infection of this patient with *P. vivax* was confirmed as described previously (Lim *et al.*, 1995).

RNA

Source of parasite used for RNA preparation was human malaria patients in India, who were confirmed by light microscopy for the presence of this parasite. The *P. vivax*-infected erythrocytes (IRBC) were purified from the patients blood by dextran sulfate gradient method (Levy and Chou, 1973). The IRBC were lysed in 1% ice-cold acetic acid to release the parasites. The total parasite RNA was then prepared according to Chomczynski and Sacchi (1987). The Poly(A)+ RNA was isolated using the oligo(dT) cellulose column from mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden) following the instructions.

PCR primers

The oligodeoxynucleotides for PCR primers were obtained from Genemed (USA) or synthesized with a model 392 automated DNA synthesizer (Applied Biosystems, Perkin Elmer, USA) using the phosporamide chemistry. The sequences are as follows; P101: 5' actagagatttcaaaactcattcc 3' (corresponding to the region from 1069 to 1092 in the 6 kb DNA sequence of *P. falciparum*, accession no. M76611), P102: 5' gtttcatatcctgcattaacatc 3' (complementary to the region from 1421 to 1443 in the 6 kb DNA sequence in *P. falciparum*, accession no. M76611), P1101: 5' ctaactattacatttattttag 3' (corresponding to the region from 283 to 304 in COIII gene fragment in *P. vivax*, accession no. D45369), P1102: 5' gattagatgtaaataagatac 3' (complementary to the region from 145 to 161 in D45369), P1103: 5' tatccgtgaattgtaaatatcat 3' (complementary to the region from 2220 to 2243 in M76611), P1104: 5' gttcctattatatggtttatgtg 3' (corresponding to the region from 4439 to 4461 in M76611).

PCR

Approximately, 8.5×10^4 saponin-lysed para-

sites were used as DNA template. For the amplification using the sets of P101 and P102, or P1102 and P1103, the 50-μl reactions were performed with 400 nM each primers, 5 units of Tth DNA polymerase (Toyobo, Osaka, Japan) at 94°C for 15 sec, 50°C for 15 sec and 72°C for 30 sec for 30 cycles. For the amplification using P1101 and P1104, the reaction (50 μl) was performed with 400 nM each primers, 1.1 mM magnesium acetate, 2 units of rTth DNA polymerase, XL (Perkin Elmer) at 94°C for 1 min for 1 cycle, 94°C for 15 sec, 48°C for 15 sec and 68°C for 5 min for 35 cycles, and 72°C for 10 min for 1 cycle. An AmpliWax PCR Gem 100 (Perkin Elmer) was used for the hot start. The thermal-programmed incubation were carried out in a GeneAmp PCR system 9600 (Perkin Elmer, USA).

Cloning of PCR fragments

The products amplified with the primer sets of P101 and P102 or P1102 and P1103 were cloned into pCRII vector (Invitrogen, San Diego, USA) according to the protocol from the supplier. The products amplified with the primers P1101 and P1104 was purified with GlassMax DNA Isolation Spin Cartridge system (Gibco BRL, Bethesda, USA), digested with *Sph* I (New England Biolabs, Massachusetts, USA), then ligated with pUC19 which was digested with *Sph* I and *Sma* I (New England Biolabs). For the transformation, *Escherichia coli* DH5α was used as a host cell.

Sequencing analysis

Cloned fragments in the recombinant plasmids were sequenced with PRISM Dye Primer Cycle Sequencing Kit (Perkin Elmer, USA), BcaBEST Dideoxy Sequencing Kit for Automated sequencer (Takara, Kyoto, Japan), PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer) as well as custom sequencing primers on a 373A automated DNA sequencer (Applied Biosystems, Perkin Elmer, USA). For the sequencing of the fragment amplified using P1101 and P1104, nested deletion mutants were prepared using Bal 31 nuclease (Promega, Madison, USA), or Exonuclease III and Mung bean nuclease (New England Biolabs) according to the standard procedures (Sambrook *et al.*, 1989) or protocol from suppliers, respectively. For the analysis of the sequencing results, packaging software in MacDNASYS (Hitachi Software, Japan), Genetyx (SDC, Japan) and Clustal V (Higgins, 1994) were used.

RT-PCR

The cDNA synthesis was carried out by using random primer and MoLV reverse transcriptase from cDNA synthesis kit (Gibco-BRL, Bethesda, USA) and 200 ng of mRNA at 42°C for 1 hr or by cDNA Synthesis Kit (Stratagene, California, USA) using oligo(dT) linker primer and 2 μg of mRNA. The 100 ng of template DNA, 200 μM each of deoxynucleotide triphosphates, 2 mM magnesium chloride, and 5 units of Taq DNA polymerase (BRL) were used for PCR using the primer set P101 and P102 (2 μM each) under following temperature profile; 94°C for 5 min for 1 cycle, then 90°C for 30 sec, 42°C for 1 min and 72°C for 1 min for 30 cycles in a Thermal Cycler (Perkin Elmer) or a PC-700 (Astec, Japan). The products were analyzed on 1.2% agarose gels.

Results and Discussion

Amplification and sequencing of the entire coding sequence of COIII gene of P. vivax

Previously, we have cloned and sequenced a part of the COIII gene of *P. vivax* (fragment A; Fig. 1. Lim *et al.*, 1995). Using the obtained sequence information as well as those of COI and cyt*b* genes of *P. falciparum* (Feagin *et al.*, 1992), we designed two sets of primers for PCR amplification of the unamplified region of the COIII gene of *P. vivax* (Fig. 1). The size of two newly amplified fragments containing parts of the *P. vivax* COIII gene were consistent with those expected from the sequence of 6 kb DNA of *P. falciparum*. Together with these results and analysis of the terminal sequence of the amplified fragments (data not shown), the gene organization of the 6 kb DNA of *P. vivax* seems to be same as those of other malaria parasites reported to date (Feagin, 1994). Using the three PCR fragments (Fig. 1), we have determined the whole region encoding COIII gene, with the size about 1 kb.

To determine the sequence of the amplified fragments, we analyzed at least 3 clones for each fragment to avoid mutations introduced during PCR amplification and the consensus sequence in at least

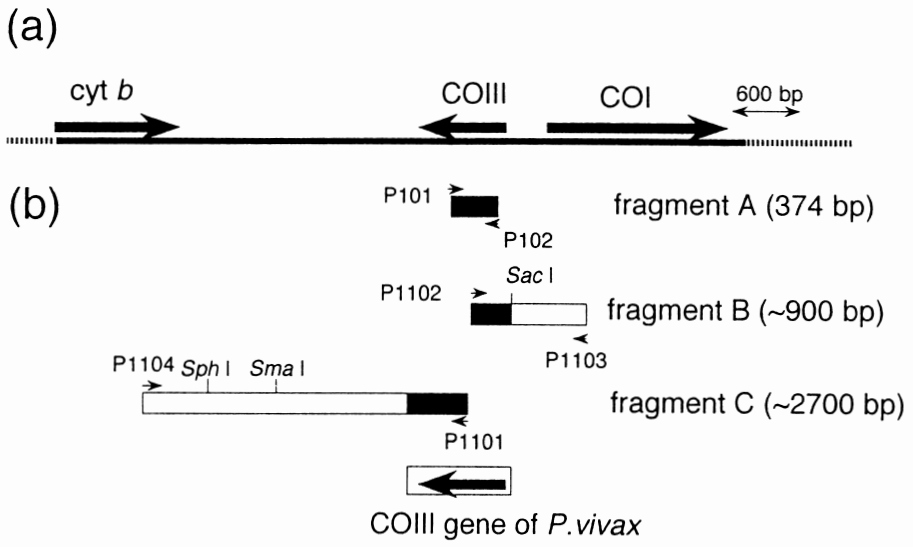

Fig. 1 Amplification of DNA fragment containing COIII gene of *P. vivax*. (a) Gene organization of the 6 kb DNA element of *P. falciparum*. Only protein coding regions are shown. (b) A schematic drawing of amplified DNA fragment of *P. vivax* and its partial restriction map. PCR primers used in this study are shown as small arrow heads. Filled boxes are regions of which sequencing was completed. The size of fragments B and C were estimated from the mobility in agarose gels because their complete sequence have not yet been obtained.

2 clones were chosen in this study. In the course of the analysis, a sequence conflict between fragments A and B at position 443 was found. In fragment A, this position was occupied by T as shown in Fig. 3 as well as the previous report. In the two clones of fragment B, it was occupied by C, although it was T in one of the clones of fragment B. This conflict did not alter the deduced amino acid sequence in the possible reading frame of COIII gene. In one of the clones for fragment C, another conflict at the position 826 was found. However this variation (A to T) may be due to the mutation during PCR amplification, although the possibility of polymorphism or divergence of the 6 kb DNA sequence in the parasite could not be neglected.

For the identification of the initiation codon of COIII gene of *P. vivax*, we compared our sequence with the corresponding region in the 6 kb DNA from *P. falciparum* (Feagin *et al.*, 1992) and *P. yoelii* (Vaidya *et al.*, 1989; Vaidya *et al.*, 1990; A. B. Vaidya, personal communication). We did not mention the *P. gallinaceum* sequence in this paper although the 6 kb DNA sequence of *P. gallinaceum* has been determined (Joseph, 1990) because the information on its COIII gene is not yet available in the public sequence database. In the case of *P. falciparum*, the ATT codon at position 96 in Fig. 2 was proposed as the initiation codon of COIII gene of *P. falciparum* (Feagin, 1992), since ATT or ATA codons are used as alternate initiation codons in several animal mitochondrial systems (Wolstenholme, 1992). In the cases of *P. vivax* and *P. yoelii*, an in-frame ATG codon was found at the position 54 in the possible reading frame (Fig. 2). This region was assigned as the coding sequence of fragment F of rRNA in ribosomal large subunit (Feagin *et al.*, 1992). The recent primer extension analysis of the COIII mRNA of *P. yoelii* (A. B. Vaidya, personal communication) showed that the 5' end of the COIII mRNA of *P. yoelii* was at the position 92 in Fig. 2. The result suggests that the initiation codons of the COIII gene of *P. yoelii* as well as *P. vivax* may be located around the position 90 in Fig. 2 and Fig. 3, similar to the case of *P. falciparum*. The identification of the initiation codon of COIII gene of *P. vivax* at the RNA or peptide level remains to be solved.

```
                    20         30         40         50         60
P.v. 10 TATACTATAACCTGTTATCCCCGGCGAACCTTCTTACCGTTATATGTTAA
P.y. 10 TATACTATAACCTGTTATCCCCGGCGAACCTTCTTACCGTTATATGTTAA
P.f. 10 TATACTATAACCTGTTATCCCCGGCGAACCTTCTTACCGTTATATGTTTA
        gene of large subunit rRNA fragment F-------->|

                    70         80         90        100        110
P.v. 61 CGGCACACAAAATCACCGTTCTTATAAATATATTTTTTATATTTAGTAAC
P.y. 61 CGGTACAAATAATCACCGTTCTTATAAATATATTTATTGTATTTAGTAAT
P.f. 61 CGGCACATTATCTCACCGTTCTTATAAATTT----ATTTTATTTAGTAAT
                                          |<-COIII mRNA(P.y.)
```

Fig. 2 Comparison of the 5'-terminal regions of COIII gene of malaria parasites. P.v.: *P. vivax* (this study); P.y.: *P. yoelii* (Vaidya *et al.*, 1989; Vaidya *et al.*, 1990; A. B. Vaidya, personal communication); P.f.: *P. falciparum* (Feagin, 1992; Feagin *et al.*, 1992). The candidates of possible translation initiation codons are underlined. The gene region of rRNA fragment F followed that of Feagin *et al.* (1992). The 5' end of COIII mRNA of *P. yoelii* determined by primer extension analysis (A. B. Vaidya, personal communication) is also shown. the numbering system is same as that in Fig. 3.

```
1                                                                GAGCTCTATATATACTATAACCTGTTATCCCCGGCGAACCTTCTTACCGTTAT    53

                                                                                                    ---------
54 ATGTTAACGGCACACAAAATCACCGTTCTTATAAATATATTTTTTATATTTAGTAACTATAATAATATTAAAGCACATTTAGTATCTTAT   143
 1  M  L  T  A  H  K  I  T  V  L  I  N  I  F  F  I  F  S  N  Y  N  N  I  K  A  H  L  V  S  Y    30

   ----P102------>                                          <--------P1102---------
144 CCTTCATTAACATCATTATATGGAACATCTTTAAAATATTTTTCTGTGGGTATCTTATTTACATCTAATCCTATTATTTTTATAATATTT   233
 31  P  S  L  T  S  L  Y  G  T  S  L  K  Y  F  S  V  G  I  L  F  T  S  N  P  I  I  F  I  I  F    60

234 GTATATTCTATTAGAGAAAGTTTTTATTCTATATTTTCATCATTAGTATCAGGAATGTTATCTATAATTATATCTGAAGCAATATTATTT   323
 61  V  Y  S  I  R  E  S  F  Y  S  I  F  S  S  L  V  S  G  M  L  S  I  I  I  S  E  A  I  L  F    90

324 ATTACATATTTTTGGGGTATTTTACATTTTAGTTTATCTCCATATCCATTATATAATGAAGGTATTATTTTAACATCATCAAGAATGTTA   413
 91  I  T  Y  F  W  G  I  L  H  F  S  L  S  P  Y  P  L  Y  N  E  G  I  I  L  T  S  S  R  M  L   120

          -------P1101------->                                                <--------P101-----
414 ATTCTAACTATTACATTTATTTTAGCAAGTGCATCATGTATGACAGCATGTTTACAATTTCTAATAGAAAAAGGAATGAGTTTAGAAATT   503
121  I  L  T  I  T  F  I  L  A  S  A  S  C  M  T  A  C  L  Q  F  L  I  E  K  G  M  S  L  E  I   150

   -----
504 TCTAGTATTGTATTTATAATATACTTACTTGGAGAATGTTTTGCATCACTTCAAACAACTGAATACTTACATTTAGGATATTGTATAAAT   593
151  S  S  I  V  F  I  I  Y  L  L  G  E  C  F  A  S  L  Q  T  T  E  Y  L  H  L  G  Y  C  I  N   180

594 GATGCTATATCTGGTACATTATTTTATTGTGTTACAGGTTTACATTTTTCACATGTTATAGTTGGTTTATTATTATTATTAATATATTTT   683
181  D  A  I  S  G  T  L  F  Y  C  V  T  G  L  H  F  S  H  V  I  V  G  L  L  L  L  L  I  Y  F   210

684 ATAAGAATAGTTGAAATGTATGATACTAATAGTGAATGGTCATATTCATTATATGGTATATCTTATATTGTATTACCTCATACTGATCAA   773
211  I  R  I  V  E  M  Y  D  T  N  S  E  W  S  Y  S  L  Y  G  I  S  Y  I  V  L  P  H  T  D  Q   240

774 ATTACAATATTATATTGGCATTTTGTTGAAATTGTATGGTTATTTATAGAGTACTTTTTCTATTCAGAATAA                    845
241  I  T  I  L  Y  W  H  F  V  E  I  V  W  L  F  I  E  Y  F  F  Y  S  E  *                   263
```

Fig. 3 Nucleotide and deduced amino acid sequences of COIII of *P. vivax*. The region from *Sac* I site in Fragment B to possible translation termination codon of this gene is shown. The regions corresponding to PCR primers (P101, P102, P1101 and P1102) are also shown. The nucleotide sequence data reported here is available in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D82020.

In our sequence, a TAA codon at the position 843 as the termination codon was found. The *P. yoelii* and *P. falciparum* sequences have the termination codons at the corresponding positions (Fig. 4). The region from 54 to 842 in Fig. 3 was tentatively assigned for the open reading frame of the *P. vivax* COIII gene. The homologies of the COIII genes between *P. vivax* and *P. yoelii* or *P. falciparum* at the

Fig. 4 Alignment of nucleotide sequences of coding region of COIII gene of *P. vivax* (P.v., this study), *P. yoelii* (P.y., A. B. Vaidya, personal communication) and *P. falciparum* (P.f., Feagin, 1992). The sequences are shown from the position corresponding to the region between possible initiation and termination codons of *P. vivax* sequence. The nucleotides identical to those of *P. vivax* sequence are shown as asterisks. The alignment was carried out by using the program Clustal V (Higgins, 1994).

nucleotide level was ~81%, ~71%, respectively (Fig. 4).

Expression of COIII gene of P.vivax *in erythrocytic stage*

To examine whether the COIII gene of *P. vivax* is expressed, RT-PCR experiment was carried out using poly(A)+ RNA from the erythrocytic stage cells of *P. vivax* (Fig. 5). The random hexamer (Fig. 5a) or oligo(dT) linker (Fig. 5b) was used as the primer for reverse transcription. In both cases, the obtained PCR products showed the predicted size from gene sequence. The results indicate that the COIII gene is expressed in the erythrocytic stages of the parasite and the COIII transcript has oligo or poly(A) sequence at the 3' terminus. These results were consistent with those of the 6 kb-DNA encoded genes from *P. yoelii* or *P. falciparum* (Suplick *et al.*, 1990; Vaidya *et al.*, 1993; Feagin and Drew, 1995). The presence of poly(A) sequence in the 6 kb DNA transcript for the protein coding regions in the other malaria parasites was also suggested (Vaidya and Arasu, 1987; Feagin and Drew, 1995).

Predicted amino acid sequence of COIII of P. vivax

The function of the COIII protein, the second-largest subunit in cytochrome *c* oxidase, is unclear. The isolation of active cytochrome *c* oxidase complex without COIII suggested that this subunit may not be involved in proton pumping (Ludwig and Schatz, 1980; Pardhasardhi *et al.*, 1991). The role of COIII protein in the assembly of the complex has been suggested (Haltia *et al.*, 1989). Further, a pool of bound lipid in COIII has been proposed to provide an oxygen reservoir in oxygen pathway (Tsukihara *et al.*, 1996).

The recent results of crystallographic studies on bacterial and bovine cytochrome *c* oxidase complex

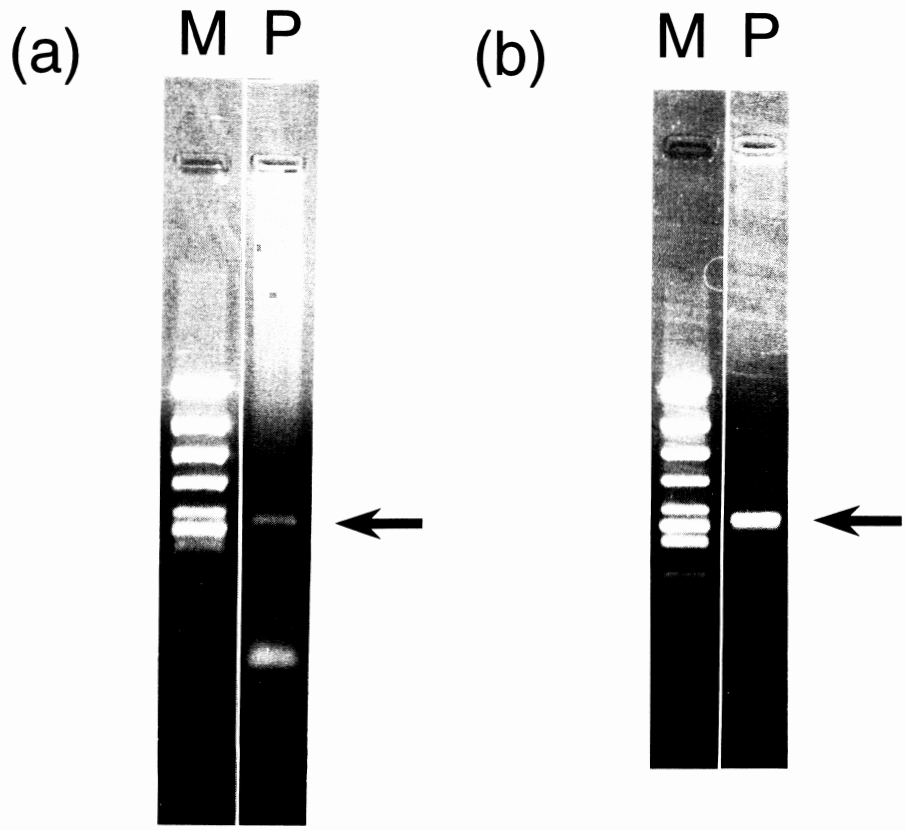

Fig. 5 Detection of *P. vivax* COIII gene transcript in the erythrocytic stages by RT-PCR; (a) Analysis of RT-PCR products using random hexamer as a primer for reverse transcription on a 1.2% agarose gel. (b) Analysis of RT-PCR products using oligo(dT) anchor primer (Stratagene) as a primer for reverse transcription on a 1.2% agarose gel. P: RT-PCR product; M: size marker (øX174 DNA/*Hinc* II digest, Nippon Gene, Japan). The locations of 374-bp products amplified by the primers P101 and P102 are shown by the arrows.

(Iwata *et al.*, 1995; Tsukihara *et al.*, 1995; Tsukihara *et al.*, 1996) revealed the higher order structure of the COIII subunit. The sequence homology of COIII sequence of *Plasmodium* to those of other organisms suggested the presence of similar, but not identical, higher order structure (Fig. 6). The amino acid homology of the COIII between *P. vivax* and *P. yoelii* or *P. falciparum* is ~82% and 73%, respectively, while the homology between *Plasmodium* and other organisms including other Apicomplexan *Theirelia parva* are less than 30%. The deletion specific for *Plasmodium* COIII were found in the N-terminal region, helix I and hydrophilic region between helices III and IV, and latter deletion was also conserved in another Apicomplexan, *T. parva*. Further, a relatively large insertion of helix VII and hydrophilic region between helices VI and VII were only found in *Plasmodium*. Such differences especially from the counterpart of human, the host organism, may relate to the different interaction(s) with other components involved in electron transport. Cytochrome *c*, the electron donor for cytochrome *c* oxidase has been suggested to be one of the candidate which interacts with COIII subunit (Iwata *et al.*, 1995). The conserved glutamate residue in helix III (Capaldi *et al.*, 1983) has been thought to be

```
P.v.          MLTAH----KITVLINIF---FIF--SNYNNI-------KAHLVSYPSLT
P.y.          I----------------------VF--SNFNNI-------KAHLVSYPSLA
P.f.          IL---------------------F--SNLSNM-------KAHLVSYPALT
T.parva       MRNSAQSYLKYINIIINIFETLYLFYSTGLDTLEYIDSTYKNFIIMYVNQY
yeast         MTHLERSRHQQHPFHMVMPSPWPI-VVSFALLS-LALSTALTMHGYIGNM
liverwort     MSV-----SQKHPFHLVDPSPWPL-LGSLGALA-STIGGVMYMHSFTGGG
human         MT------HQSHAYHMVKPSPWPL-TGALSALL-MTSGLAMWFH-FHSM-
              +                      +       +             +
                                  <------helix I------>

P.v.          SLYGTSLKYFSVGILFTSNPIIFIIFVYSIRESFYSIFSSLVSGMLSIII
P.y.          SLYGTSLKYFSVGILFTFNPIILLIFVYSIRESLYSTFSSLASGMLSIII
P.f.          SLYGTSLKYFSVGMLFTFNPMILLMFVYSIRESFYSVFSSLTSGMLSIMM
T.parva       LLYGTTLKYLSVGEFFMNS---LTIFINSIREIMTSTTMVMYAIFGMFIF
yeast         NMVYLAL-FVLLTSSILWFRDIVAEATYLGDHTM-AVRKGINLGFLMFVL
liverwort     TLLCLGL-GMILYTMFVWWRDVIRESTYEGHHTF-VVQLGLRYGIILFIV
human         TLLMLGL-LTNTLTMYQWWRDVTRESTYQGHHTP-PVQKGLRYGMILFIT
               +    *                          +        +      ++
              <------helix II----------->      <------helix III-

P.v.          SEAILFITYFWGILHFSLSPYPLYNE-----GI-ILTSSRMLILTITFIL
P.y.          SEALLFLTYFWGILHFCLSPYPLYDE-----GI-IITSSRMLILTITFIL
P.f.          SEALLFFTYFWGMLHFSLSPYPLSNE-----GI-IITSSRMLILTITFML
T.parva       SEILVFSTFIWGYFHLRLSN-PILLA-----EL-NVEA----YLQISDVL
yeast         SEVLIFAGLFWAYFHSAMSPDVTLGACWPPVGIEAVQPTELPLLNTIILL
liverwort     SEVMFFLAFFWAFFHSSLAPTVEIGAIWPPKGISVLDPWGIPFLNTLILL
human         SEVFFFAGFFWAFYHSSLAPTPQLGGHWPRTGITPLNPLEVPLLNTSVLL
              *#   *   +*+  *  ++              +  + +     *    +*
              ---------------->                          <---------

P.v.          ASASCMTACLQFLIEKGMSLEISSIVFIIYLLGECFASLQTTEY-LHLGY
P.y.          ASASCMTACLQFLIEKGMSFEISSIVCIIYLLGECFASLQTTEY-LHLSY
P.f.          ASASCMTACLQVFMEKGMSFEISSIICMMYLLGECFASLQTTEY-LHLSY
T.parva       NTGSILVSIILHRVQESANFETDFFMEQLLLIGFIFLSLQNDEYSLILSY
yeast         SSGATVTYSHHALIAGNRNKALSGLL-ITFWLIVIFVTCQYIEY-TNAAF
liverwort     SSGAAVTWAHHAILAGLKQQAVYALI-ATVFLALVFTGFQGIEY-IEAPF
human         ASGVSITWAHHSLMENNRNQMIQALL-ITILLGLYFTLLQASEY-FESPF
               ++  +       +          ++     +   *   *  **     +
              -helix IV---->   <----------helix V----------->

P.v.          CINDAISGTLFYCVTGLHFSHVIVGLLLLLI--YFIRIVEMYDTNSEWSY
P.y.          YINDAVLGTLFYCVTGLHFTHVIVGLILLLI--YFIRIVDQYDVNTEWSY
P.f.          HMNDTVYTTLFYCVTGLHFSHVVMGLLLLMM--YFMRMMEMYDTSTEWFM
T.parva       -VNNYWMTLYFFILTGLHSLHVCAGGIFVLIQSYF------YEGDGSQRD
yeast         TISDGVYGSVFYAGTGLHFLHMVMLAAMLGV-NYWRMRNYHLTAGHH---
liverwort     TISDGIYGSTFFLATGFHGFHVIIGTIFLII-CGIRQYLGHFTPKHH---
human         TISDGIYGSTFFVATGFHGLHVIIGSTFLTI-CFIRQLMFHFTSKHH---
               +++      *+  ***+  *+       + +
                  <---------helix VI--------------->

P.v.          SLYGISYIVLPHTDQITILYWHFVEIVWLFIEYFFY---SE
P.y.          SYIGISYVVFTHTDQMTILYWHFVEIVWLFIEFFFY---SE
P.f.          NSFGMSYIVMPHTDQITILYWHFVEMIWLFMEFLFY---SE
T.parva       EEFNAG------------VYWHFVEMIWIALTMLLF---LA
yeast         ----VGY-------ETTIIYTHVLDVIWLFLYVTFYWWGV-
liverwort     ----FGF-------EAAAFYWHFVDVVWLFLFVSIYWWGGN
human         ----FGF-------EAAAWYWHFVDVVWLFLYVSIYWWGS-
                +           * * ++++*+ +   ++
              <---------helix VII------------>
```

Fig. 6 Comparison of amino acid sequences of COIII from *P. vivax* (P.v., this study), *P. yoelii* (P.y., A. B. Vaidya, personal communication) *P. falciparum* (P.f., Feagin, 1992), *Theirelia parva* (T. parva, Kairo *et al.*, 1994), *Saccharomyces cerevisiae* (yeast, Thalenfelt and Tzagoloff, 1980), liverwort (Oda *et al.*, 1992) and human (Anderson *et al.*, 1981). Alignment was carried out by using the program Clustal V (Higgins, 1994). *; conserved residues between the six organisms. #; the conserved dicyclohexyl carbodiimide binding glutamate residue. +; semi-conserved residues. The transmembrane helical regions (helices I to VII) in human sequence predicted by the crystal structure of bovine counterpart (Tsukihara *et al.*, 1996) are also shown.

Table 1 Amino acid identity between COIII peptides from various organisms (%)

	P.y.	P.f.	T. parva	yeast	liverwort	human
P.v.	81.8	73.0	28.1	24.0	24.5	27.4
	P.y.	76.7	29.2	26.4	26.3	26.9
		P.f.	27.7	25.4	23.3	26.7
			T. parva	22.2	24.0	24.7
				yeast	43.3	43.5
					liverwort	62.3

The comparison was carried out using the program Maximam Matching in Genetyx software package (SDC). The sequence data were from *P. vivax* (P.v., this study), *P. yoelii* (P.y., A.B. Vaidya, personal communication), *P. falciparum* (P.f., Feagin, 1992), *Theirelia parva* (T. parva, Kairo *et al.*, 1994), *Saccharomyces cerevisiae* (yeast, Thalenfelt and Tzagoloff, 1980), liverwort (Oda *et al.*, 1992) and human (Anderson *et al.*, 1981).

functionally important in proton translocation since its modification by dicyclohexyl carbodiimide inhibits proton pumping in cytochrome *c* oxidase (Haltia *et al.*, 1991). Recent crystallographic studies on bacterial COIII suggested that the role of this residue may be structural (Iwata *et al.*, 1995). This glutamate residue is also conserved in *P. vivax* as well as other *Plasmodium* species reported to date.

Concluding Remarks

We have determined the COIII gene of *P. vivax* to elucidate the detail character of respiratory chain components of malaria parasites. Because of the multicopy number of the 6 kb DNA unit in the malaria parasite (Feagin, 1994), the 6 kb DNA sequence is thought to be a good candidate for the PCR diagnosis and identification of species. The sequence information of the COIII gene presented in this study is useful for such a diagnostic application as published elsewhere (Lim *et al.*, 1996).

The results obtained in this study revealed several specific features of the COIII peptide of malaria parasites. Such features might be a possible target for chemotherapy. The COIII subunit is thought to interact with other subunits of the enzyme as well as other components. Therefore the other components encoded in both the 6 kb and genomic DNA of *Plasmodium* would be critical to identify the specific feature of respiratory chain of malaria parasites. Such studies are now in progress.

Acknowledgments

The authors would like to thank Dr. A. B. Vaidya, Allegheny University, USA for the update sequence and primer extension data of the COIII gene of *P. yoelii* and the Director, Institute for Medical Research, Kuala Lumpur, Malaysia for permission to publish.

References

1) Aldritt, S. M., Joseph, J. T., Wirth, D. F. (1989): Sequence identification of cytochrome *b* in *Plasmodium gallinaceum*. Mol. Cell. Biol., 9, 3614–3620.
2) Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R. and Young, I. G. (1981): Sequence and organization of the human mitochondrial genome. Nature, 290, 457–465.
3) Capaldi, R. A., Malatesta, F. and Darley-Usmar, V. M. (1983): Structure of cytochrome *c* oxidase. Biochim. Biophys. Acta, 726, 135–148.
4) Chomczynski, P. and Sacchi, N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162, 156–159.
5) Divo, A. A., Geary, T. G. and Jensen, J. B. (1985): Oxygen- and time-dependent effects of antibiotics and selected mitochondrial inhibitors on *Plasmodium falciparum* in culture. Antimicrob. Agents Chemother.,

27, 21–27.

6) Divo, A. A., Geary, T. G., Jensen, J. B. and Ginburg, H. (1985b): The mitochondrion of *Plasmodium falciparum* visualized by rhodamine 123 fluorescence. J. Protozool., 32, 442–446.
7) Feagin, J. E. (1992): The 6-kb element of *Plasmodium falciparum* encodes mitochondrial cytochrome genes. Mol. Biochem. Parasitol., 52, 145–148.
8) Feagin, J. E. (1994): The extrachromosomal DNAs of apicomplexan parasites. Ann. Rev. Microbiol., 48, 81–104.
9) Feagin, J. E. and Drew, M. E. (1995): *Plasmodium falciparum*: alterations in organelle transcript abundance during the erythrocytic cycle. Exp. Parasitol., 80, 430–440.
10) Feagin, J. E., Werner, E., Gardner, M. J., Williamson, D. H. and Wilson, R. J. (1992): Homologies between the contiguous and fragmented rRNAs of the two *Plasmodium falciparum* extrachromosomal DNAs are limited to core sequences. Nucleic Acids. Res., 20, 879–887.
11) Fry, M. and Beesley, J. E. (1991): Mitochondria of mammalian *Plasmodium* spp. Parasitology, 102, 17–26.
12) Geary, T. G. and Jensen, J. B. (1983): Effects of antibiotics on *Plasmodium falciparum* in vitro. Am. J. Trop. Med. Hyg., 32, 221–225.
13) Ginsburg, H., Divo, A. A., Geary, T. G., Boltand, M. T. and Jensen, J. B. (1986): Effects of mitochondrial inhibitors of intraerythrocytic *Plasmodium falciparum* in in vitro cultures. J. Protozool., 33, 121–125.
14) Gutteridge, W. E., Dave, D. and Richards, W. H. (1979): Conversion of dihydroorotate to orotate in parasitic protozoa. Biochim. Biophys. Acta, 582, 390–401.
15) Haltia, T., Finel, M., Harms, N., Nakari, T., Raitio, M., Wikström, M. and Saraste, M. (1989): Deletion of the gene for subunit III leads to defective assembly of bacterial cytochrome oxidase. EMBO J., 8, 3571–3579.
16) Haltia, T., Saraste, M. and Wikström, M. (1991): Subunit III of cytochrome *c* oxidase is not involved in proton translocation: a site-directed mutagenesis study. EMBO J., 10, 2015–2021.
17) Higgins, D. G. (1994): CLUSTAL V: multiple alignment of DNA and protein sequences. Methods Mol. Biol., 25, 307–318.
18) Iwata, S., Ostermeier, C., Ludwig, B. and Michel, H. (1995): Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. Nature, 376, 660–669.
19) Joseph, J. T. (1990): The mitochondrial microgenome of malaria parasites. PhD thesis. Harvard Medical School, Cambridge.
20) Joseph, J. T., Aldritt, S. M., Unnasch, T., Puijalon, O. and Wirth, D. F. (1989): Characterization of a conserved extrachromosomal element isolated from the avian malarial parasite *Plasmodium gallinaceum*. Mol. Cell. Biol., 9, 3621–3629.
21) Kairo, A., Fairlamb, A. H., Gobright, E. and Nene, V. (1994): A 7.1 kb linear DNA molecule of *Theileria parva* has scrambled rDNA sequences and open reading frames for mitochondrially encoded proteins. EMBO J., 13, 898–905.
22) Krungkrai, J. (1995): Purification, characterization and localization of mitochondrial dihydroorotate dehydrogenase in *Plasmodium falciparum*, human malaria parasite. Biochim. Biophys. Acta, 1243, 351–360.
23) Levy, M. R. and Chou, S. C. (1973): Activity and some properties of an acid proteinase from normal and *Plasmodium berghei* infected red cells. J. Parasitol., 59, 1064–1070.
24) Lim, P. K. C., Kita, K., Tan, S. K., Furuta, T., Kojima, S., Aoki, T., Watanabe, Y. and Mak, J. W. (1995): Mitochondrial cytochrome *c* oxidase III (COIII) gene of *Plasmodium vivax*. Jpn. J. Parasitol., 44, 40–43.
25) Lim, P. K. C., Tan, S. K., Kita, K., Kojima, S., Watanabe, Y. and Mak, J. W. (1996): Species-specific diagnosis of human malaria by the polymerase chain reaction. IMR J., 1, *in press*.
26) Ludwig, B. and Schatz, G. (1980): A two-subunit cytochrome *c* oxidase (cytochrome aa3) from *Paracoccus dentrificans*. Proc. Natl. Acad. Sci., USA, 77, 196–200.
27) Oda, K., Yamato, K., Ohta, E., Nakumura, Y., Takemura, M., Nozato, N., Akashi, K., Kanegae, T., Ogura, Y., Kohchi, T. and Ohyama, K. (1992): Gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* mitochondrial DNA. A primitive form of plant mitochondrial genome. J. Mol. Biol., 223, 1–7.
28) Pardhasaradhi, K., Ludwig, B. and Hendler, R. W. (1991): Potentiometric and spectral studies with two-subunit cytochrome aa_3 from *Paracoccus denitrificans*. Comparison with the 13-subunit beef heart enzyme. Biophys. J., 60, 415–423.
29) Preiser, P. R., Wilson, R. J., Moore, P. W., McCready, S., Hajibagheri, N. A. N., Blight, K. J., Starth, M. and Williamson, D. H. (1996): Recombination associated with replication of malarial mitochondrial DNA. EMBO J., 15, 684–693.
30) Sambrook, J., Fritsch, T. and Maniatis, T. (1989): Molecular Cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York, 15.20–15.26.
31) Scheibel, L. and Pflaum, W. K. (1970): Cytochrome oxidase activity in platelet-free preparations of *Plasmodium falciparum*. J. Parasitol., 56, 1054.
32) Sherman, I. W. (1979): Biochemistry of *Plasmodium* (Malarial parasites). Microbiol. Rev., 43, 453–495.
33) Suplick, K., Morrisey, J. and Vaidya, A. B. (1990): Complex transcription from the extrachromosomal DNA encoding mitochondrial functions of *Plasmodium yoelii*. Mol. Cell. Biol., 10, 6381–6388.
34) Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1995): Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å. Science, 269, 1069–1074.

35) Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. and Yoshikawa, S. (1996): The whole structures of the 13-subunit oxidazed bovine cytochrome *c* oxidase at 2.8 Å. Science, 272, 1136–1144.

36) Thalenfeld, B. E. and Tzagoloff, A. (1980): Assembly of the mitochondrial membrane system. Sequence of the oxi 2 gene of yeast mitochondrial DNA. J. Biol. Chem., 255, 6173–6180.

37) Vaidya, A. B., Akella, R. and Suplick, K. (1989): Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. Mol. Biochem. Parasitol., 35, 97–107.

38) Vaidya, A. B., Akella, R. and Suplick, K. (1990): Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite (Corrigendum). Mol. Biochem. Parasitol., 39, 295–296.

39) Vaidya, A. B. and Arasu, P. (1987): Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed. Mol. Biochem. Parasitol., 22, 249–257.

40) Vaidya, A. B., Lashgari, M. S., Pologe, L. G. and Morrisey, J. (1993): Structural features of *Plasmodium* cytochrome *b* that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. Mol. Biochem. Parasitol., 58, 33–42.

41) Wilson, R. J., Fry, M., Gardner, M. J., Feagin, J. E., Williamson, D. H. (1992): Subcellular fractionation of the two organelle DNAs of malaria parasites. Curr. Genet., 21, 405–408.

42) Wolstenholme, D. R. (1992): Animal mitochondrial DNA: structure and evolution. Int. Rev. Cytol., 141, 173–216.