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Mitochondrial RNA polymerase is an essential enzyme in erythrocytic stages of *Plasmodium falciparum*

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Abstract

We have shown that transgenic *Plasmodium falciparum* parasites expressing the yeast DHODH (dihydroorotate dehydrogenase) are independent of the mtETC (mitochondrial electron transport chain), suggesting that they might not need the mitochondrial genome (mtDNA), since it only encodes three protein subunits belonging to the mtETC and fragmentary ribosomal RNA molecules. Disrupting the mitochondrial RNA polymerase (mtRNAP), which is critical for mtDNA replication and transcription, might then cause the generation of a ρ^0 parasite line lacking mtDNA. We made multiple attempts to disrupt the mtRNAP gene by double crossover recombination methods in parasite lines expressing yDHODH either episomally or integrated in the genome, but were unable to produce the desired knockout. We verified that the mtRNAP gene was accessible to recombination by successfully integrating a triple HA tag at the 3' end via single cross-over recombination. These studies suggest that mtRNAP is essential even in mtETC-independent *P. falciparum* parasites.

Keywords

malaria; *Plasmodium falciparum*; ρ^0 ; mitochondrial RNA polymerase; gene knockout

Previous studies from our laboratory have revealed that the essential function of the mitochondrial electron transport chain (mtETC) is to regenerate ubiquinone to serve dihydroorotate dehydrogenase (DHODH), which is required for pyrimidine *de novo* biosynthesis [1]. Transgenic parasites (D10 and 3D7 lines), in which the yeast DHODH (ubiquinone-independent) functionally replaced the parasite DHODH, were resistant to all the mtETC inhibitors, suggesting that these transgenic parasites are independent of the mtETC [2]. Malaria parasites possess a highly conserved 6 kb mtDNA (mitochondrial DNA); each species has approximately 30–100 copies of the 6 kb DNA, which are arranged in head to tail tandem arrays [3, 4]. The mtDNA encodes just three proteins, cytochrome *b* (cytb), and subunit I and III of cytochrome *c* oxidase (coxI and coxIII), which are components of the mtETC. In addition, the parasite mtDNA specifies 34 small RNA molecules, which together constitute the small and large subunits of mitochondrial ribosomal RNA [5–7]. Thus, the entire mitochondrial genetic system appears to be designed

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to provide just three components of mtETC, and it is possible to dispense with a functional mtETC through a metabolic bypass provided by cytosolic DHODH. This raised possibility of generating parasite lines that have lost the mtDNA (ρ^0 parasites) as an aid to investigate mitochondrial functions in malaria parasites.

Many organisms can be induced to become ρ^0 cells by a variety of agents. In *Saccharomyces cerevisiae*, extensive treatment with ethidium bromide induced respiration deficient petite cells; ethidium bromide eliminated the mtDNA by altering the mitochondrial DNA and RNA synthesis without affecting the nuclear DNA [8]. In yeast, active membrane solvents, such as ethanol, isopropanol, and sodium dodecyl sulfate, can also induce petite mutants by damaging the mitochondrial membranes [9]. Long-term exposure (4–6 weeks) to ethidium bromide also induced ρ^0 cells in human osteosarcoma cell line 143B.TK⁻ [10]. Recently, Kukat *et al.* developed an enzymatic approach to induce ρ^0 cells in mouse, rat and human cell lines by targeting a restriction endonuclease to the mitochondria that cleaved the mtDNA [11]. The ρ^0 cells generated by these various methods are oxidative phosphorylation deficient and may require additional nutrients (such as glucose, uridine or pyruvate), but clearly the mtDNA is otherwise not indispensable for cell survival and growth.

In addition to transcription, mitochondrial RNA polymerase (mtRNAP) is likely involved in mtDNA replication by synthesizing RNA primers required for initiating DNA replication at the leading and/or lagging DNA strands (Okazaki fragments) [12]. Thus, successful disruption of mtRNAP should eventually result in the elimination of the mtDNA. In this study, we sought to knock out the *P. falciparum* mtRNAP in an yDHODH-positive background, thereby causing the elimination of the mtDNA. The putative *P. falciparum* mitochondrial RNA polymerase (PF11_0264) is a nuclearly encoded protein with 1531 amino acids and is the only member of the T3/T7 phage-like RNA polymerase family found in the *Plasmodium* genome [13]. The T3/T7 phage-like RNA polymerase family includes virtually all known mitochondrial RNA polymerases [14]. PfmtRNAP has a predicted mitochondrial targeting sequence at the amino-terminus and has conserved domains shared by mitochondrial RNA polymerases in the carboxyl-terminal portion of the protein [13].

We used three sets of knockout vector constructs and *P. falciparum* lines in our attempts to disrupt the mtRNAP gene. Initially, we tried to knock out the mtRNAP gene in the first available yDHODH-expressing *P. falciparum* line, 3D7-yDHODH-GFP [1], in which yDHODH was expressed from the episomal plasmid pHH-yDHOD-GFP (Supplementary Fig. 1). Since pHH-yDHOD-GFP shares many elements with the knockout plasmids (pCC1, pCC4; Supplementary Fig. 1), to reduce the chances of selecting recombination between the transgene and knockout plasmids, we generated a new plasmid pJK86 (Supplementary Fig. 1) by replacing the two sections of pCC4 that are 3' to the *bsd* selectable marker gene with elements having unrelated sequences: heat shock protein 86 3' untranslated region (UTR) replaced histidine rich protein 2 3' UTR, and pJK4 (R6K plasmid replication origin + kanamycin resistance gene) replaced pGEM3Z (pUC origin + ampicillin resistance gene) (Supplementary Fig. 1; see Supplementary data for methods). The elimination of homologous sequence on the 3' side of the positive selectable marker should prevent one route of escape from negative selection via exchange of positive selectable markers between pJK86 and pHH-yDHOD-GFP by homologous recombination events and consequent separation of the *bsd* positive selection marker from the negative selection gene *yFCU*, yeast fusion gene of cytosine deaminase/uracil phosphoribosyl transferase (see Supplementary Fig. 1).

To confirm that pJK86 can propagate in malaria parasites, we transfected the 3D7 wild type parasites with this plasmid. Blasticidin (BS) resistant parasites were achieved a few weeks following transfection, suggesting that pJK86 is suitable for transfections (data not shown).

We then cloned the 5' and 3' flanking homologous sequences of the mtRNAP gene in the pJK86 plasmid, yielding the knockout construct pJK86 Δ RNAP (see Supplementary data for detailed methods). We transfected 3D7-yDHODH-GFP parasites with pJK86 Δ RNAP and maintained the culture under BS (for pJK86 Δ RNAP) plus WR99210 (WR, for pHH-yDHODH-GFP) selections. BS- and WR-resistant parasites were observed 6 weeks following transfection, which were then treated with 2 μ M 5-FC (5-fluorocytosine) for negative selection. About three weeks later, 5-FC-resistant parasites were generated. Theoretically, only knockout parasites which have undergone double crossover recombination to replace a significant portion of the mtRNAP gene with the *bsd* cassette, followed by the loss of the remnant plasmid containing the yFCU negative selection gene cassette would exhibit resistance to the toxic precursor compound 5-FC (Figure 1). However, PCR analysis showed that the mtRNAP gene was intact in these parasites, which we designated BS+WR+5FC parasites (Figure 2A). To attempt to ascertain the reason for 5-FC resistance, we recovered plasmid DNA from the BS+WR+5FC parasites. The yFCU gene was amplified by PCR from this plasmid, and the sequence of the entire yFCU cassette, including the heat shock protein 86 5' UTR (the promoter), the yFCU gene and the *P. berghei* dihydrofolate reductase-thymidylate synthase 3' UTR (the terminator), was confirmed to contain no mutations or deletions (data not shown). It seems that the parasites have managed to avoid 5-FC toxicity by an unknown mechanism, xx by shutting down the yFCU gene expression either transcriptionally or translationally. Mechanisms for that are quite complex, exemplified by var gene expression. It was also interesting to note that the two episomal plasmids, pHH-yDHODH-GFP and pJK86 Δ RNAP, had recombined to produce a larger combined plasmid. The recovered plasmid had the size of sum of the the two plasmids (pHH-yDHODH-GFP and pJK86 Δ RNAP) in total and was resistant to ampicillin and kanamycin simultaneously (data not shown). Although pJK86 contained a reduced number of identical sequence elements in common with pHH-yDHODH-GFP relative to pCC4, several long identical elements remained (Supplementary Fig. 1), evidently allowing integration by single crossover homologous recombination. We also cycled BS off-and-on three times and resumed 5-FC treatment after each cycling. In each cycle, we took BS selection off for 4 weeks and added it back on to re-select BS resistant parasites. Drug off-and-on cycling is a common but time-consuming strategy to increase the percentage of the parasites in which plasmids have integrated by single or double crossover recombination. However, none of these drug cycling trials yielded detectable disruption of the mtRNAP gene, as shown by PCR analysis (Figure 2A).

To avoid plasmid recombination issues, we next tried to knock out the mtRNAP gene in D10-yDHODH parasites that contain a copy of the yDHODH gene integrated into the non-essential type II vacuolar proton-pumping pyrophosphatase gene [15]. We transfected D10-yDHODH parasites with the knockout construct pCC1 Δ RNAP, containing the same 5' and 3' flanking homologous sequences as in the first construct (Supplementary data). WR-resistant parasites were achieved after several weeks and 5-FC was added to the culture for negative selection. Subsequently, we cycled WR off-and-on three times and applied 5-FC treatment after each cycling. Drug resistant parasites were obtained each time after 5-FC treatment, however, none of these parasite cultures showed evidence of an mtRNAP gene disruption (Figure 2A).

A third knockout construct was prepared using the pUF1 plasmid, which contains an endogenous yDHODH expression cassette serving as the positive selection marker [15], and the same 5' and 3' flanking homologous sequences as in the previous constructs. This knockout construct was transfected into *P. falciparum* D10 parasites, and selected with DSM1, a specific inhibitor of *P. falciparum* DHODH [16]. DSM1 resistant parasites were achieved several weeks after transfection. We added 5-FC directly to the DSM1-selected

culture and to DSM1 off-and-on cycled cultures (for three cycles), but none of these trials yielded mtRNAP knockout parasites (Figure 2A).

The results of all three series of knockout experiments indicate that the *P. falciparum* mtRNAP gene is refractory to disruption. To rule out the possibility that the failure of gene disruption was due to the non-accessibility of the mtRNAP gene, we performed an integration experiment to fuse a triple HA (haemagglutinin epitope) tag at the 3' end of the mtRNAP gene by single crossover recombination (Figure 1, Figure 2B). We cloned the 906 bp fragment from the 3' end of the mtRNAP gene into a modified pUF1 plasmid in which a sequence encoding a triple HA tag had been inserted in front of the *P. berghei* dihydrofolate reductase-thymidylate synthase 3' UTR terminator (Supplementary data). We transfected D10 wild type parasites with the plasmid pUF1-3'RNAPHA. We achieved DSM1-resistant parasites following transfection and cycled DSM1 off-and-on three times. PCR analysis showed that the DSM1-cycled parasites had integrated the pUF1-3'RNAPHA plasmid by single crossover recombination (Figure 2B), suggesting that the mtRNAP gene locus is accessible for genetic recombination. With the HA tagged parasites in hand (D10-pUF1-3'RNAPHA), we tried to check the localization of the mtRNAP protein by immunofluorescence assay (IFA), but failed to detect any signal in the parasites (data not shown). Nor did Western Blot analysis detect the expected band of the HA-tagged mtRNAP using the highest sensitivity reagents (data not shown). Thus, both IFA and Western blot indicated that the expression level of the mtRNAP was very low. This low level of expression was also noted by the previous study that identified mtRNAP [13].

In summary, we tried to knock out the mitochondrial RNA polymerase gene in the mtETC-independent *Plasmodium falciparum* parasites, hoping to generate ρ^0 malaria parasites, but the gene appears to be essential in blood stage parasites. The failure to disrupt the gene does not appear to be due to technical problems. We have successfully knocked out a series of nuclear-encoded mitochondrial genes in *P. falciparum*, including many tricarboxylic acid pathway genes, using the same approaches based on gene replacement using pCC1, pCC4, and pUF1 plasmid constructs (Hangjun Ke, unpublished data). In this study, we performed the knockout experiments in yDHODH transgenic parasites derived from two different cell lines (3D7 and D10), suggesting that the results are not dependent on a specific cell type. We cycled each of the positive selection drugs, blasticidin, WR992210, or DSM1, three times to increase the probability of parasites to have undergone plasmid integration events by homologous recombination, and then performed 5-FC selections. Thus, the failure to produce the desired knockout does not seem to be due to insufficient time allowed for gene recombination to occur. Nor does the failure appear to be due to the non-accessibility of the mtRNAP gene, as we successfully integrated a triple HA tag at the 3' end of the mtRNAP gene by single crossover recombination.

We conclude that mitochondrial RNA polymerase is an essential gene even in mtETC-independent parasites. Why do parasites still need mtRNAP when they do not need mtETC activity? It is possible that in addition to transcription and priming mtDNA replication, mtRNAP has some critical, as yet unknown, alternative functions. A second possibility is alternate splicing or start site that produces an alternate form of the polymerase with possible targeting to a different compartment, such as the nucleus, as apparently occurs in mammals [17]. The alternate transcript of the mtRNAP gene was shown to transcribe certain nuclear mRNAs in humans and rodents. A third possibility is that mtDNA gene products, including cytb, coxI, coxIII and ribosomal RNAs, have other important functions, in addition to generating or participating in the mtETC. For example, mtDNA gene products might play a role in proper mitochondrial morphogenesis. Appropriate assembly of mitochondrial inner membrane complexes has been shown in other systems to be important for supercomplex formation and aspects of mitochondrial morphogenesis (for example, see

[18, 19]). Thus, mtETC components may be critical for functions other than electron transport.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BS	blasticidin
DHODH	dihydroorotate dehydrogenase
yDHODH	yeast dihydroorotate dehydrogenase
mtETC	mitochondrial electron transport chain
mtRNAP	mitochondrial RNA polymerase
HA	hemagglutinin
mtDNA	mitochondrial DNA
cytb	cytochrome b
coxI	cytochrome c oxidase subunit I
coxIII	cytochrome c oxidase subunit III
5-FC	5-fluorocytosine
yFCU	yeast fusion gene of cytosine deaminase/uracil phosphoribosyl transferase
DSM1	5-methyl[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)naphthalen-2-ylamine (a DHODH inhibitor)
dhfr	dihydrofolate reductase
bsd	blasticidin deaminase
UTR	untranslated region (of a gene)
WR	WR99210 (4,6-diamino-1,2-dihydro-2,2-dimethyl-1-[(2,4,5-trichlorophenoxy)propyloxy]-1,3,5-triazine)

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Highlights

- The mitochondrial RNA polymerase is essential even when mtETC activity is not.
- A novel R6K-based plasmid propagates in *P. falciparum*.
- Negative selection is readily subverted.

Figure 1.

Schematic diagrams of double cross-over recombination and 3' end triple HA integration in the mitochondrial RNA polymerase gene locus. (A) The genotypes of wild type and knockout. The mtRNAP gene is 4596 bp long. The 5' homologous flanking sequence (5'f), amplified from positions 1733 bp to 2624 bp, and the 3' homologous flanking sequence (3'f), positions 3687 bp to 4597 bp, were cloned into pJK86, pCC1 and pUF1 plasmids individually. "Drug cassette" stands for bsd in pJK86, hdhfr in pCC1, and yDHODH in pUF1, and includes parasite-derived 5' and 3' UTRs providing the promoter and terminator to drive expression. Through double cross-over recombination, a fragment of the mtRNAP (positions 2625 to 3686 bp) would be replaced with the drug cassette. The arrows indicate primers used to check the genotypes (see Fig. 2), which amplify a 2.8 kb band from the wild type genomic DNA, and would amplify a 3.8 kb (in the case of the pJK86 construct), 3.9 kb (pCC1 construct) or 4.3 kb (pUF1 construct) band from the genomic DNA of a corresponding knockout line. (B) The genotypes of wild type and 3' triple HA integration. The 3' fragment (906 bp), positions 3687 to 4593 bp, was cloned into the modified pUF1 plasmid, pUF1HA (S.M. Ganesan, unpublished), which contains a triple HA tag immediately preceding a *P. berghei* dihydrofolate reductase-thymidylate synthase 3' UTR terminator. Through single cross-over recombination, the entire plasmid would be integrated at the 3' end of the mtRNAP gene. The arrows indicate primers used to check the genotypes of wild type and prospective 3' HA integration lines (see Fig. 2), which amplify no band from the wild type DNA, but a 1.1 kb band from genomic DNA having the 3' HA integration. The forward primer is located in front of the 3' fragment in the genomic sequence while the reverse primer is located after the HA tag in the plasmid backbone. HA, hemagglutinin; RNAP, RNA polymerase.

Figure 2.

The mtRNAP gene could not be deleted. (A) Diagnostic PCR checking the genotypes of genomic DNA from the transgenic and wild type parasites. Lanes 1 to 4 are from the pJK86 Δ RNAP/3D7-yDHODH-GFP transgenic parasites. Lane 1: WR+BS+5-FC selected. Lane 2: WR +BS (+1 cycle)+5-FC selected. Lane 3: WR +BS (+2 cycles)+5-FC selected. Lane 4: WR +BS (+3 cycles)+5-FC selected. Lanes 5 to 8 are from the pCC1 Δ RNAP/D10-yDHODH transgenic parasites. Lane 5: WR+5-FC selected. Lane 6: WR (+1 cycle)+5-FC selected. Lane 7: WR (+2 cycles)+5-FC selected. Lane 8: WR (+3 cycles)+5-FC selected. Lanes 9 to 12 are from the pUF1 Δ RNAP/D10 transgenic parasites. Lane 9: DSM1+5-FC selected. Lane 10: DSM1 (+1 cycle)+5-FC selected. Lane 11: DSM1 (+2 cycles)+5-FC selected. Lane 12: DSM1 (+3 cycles)+5-FC selected. Lane 13: 3D7 wild type. Lane 14: D10 wild type. Lane 15: No DNA control. The primer positions are shown in Figure 1(A) and sequences are shown in Supplementary Table 1. (B) PCR checking the genotypes of the DNAs from the 3' HA integrated parasites. Lane 1: DSM1 +1 cycle-selected. Lane 2: DSM1 +2 cycle-selected. Lane 3: DSM1 +3 cycle-selected. Lane 4: D10 wild type. Lane 5: pUF13'RNAPHA plasmid. Lane 6: No DNA control. The primer positions are shown in Figure 1(B) and sequences are shown in Supplementary Table 1.