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Polysome profiling of the malaria parasite *Plasmodium falciparum*

Joshua R. Lacsina^{a,b,*}, Gregory LaMonte^{c,d,*}, Christopher V. Nicchitta^{a,b,§}, and Jen-Tsan Chi^{c,d,§}

Joshua R. Lacsina: jrl3@duke.edu; Gregory LaMonte: greg.lamonte@duke.edu; Christopher V. Nicchitta: c.nicchitta@cellbio.duke.edu; Jen-Tsan Chi: chi00002@mc.duke.edu

^a Department of Pathology, Duke University School of Medicine, Durham, North Carolina, United States of America, 27710

^b Department of Cell Biology, Duke University School of Medicine, Durham, North Carolina, United States of America, 27710

^c Department of Molecular Genetics and Microbiology, Duke University School of Medicine, Durham, North Carolina, United States of America, 27710

^d The Institute for Genome Sciences and Policy, Duke University School of Medicine, Durham, North Carolina, United States of America, 27710

Abstract

In the malaria parasite *Plasmodium falciparum*, global studies of translational regulation have been hampered by the inability to isolate malaria polysomes. We describe here a novel method for polysome profiling in *Plasmodium falciparum*, a powerful approach which allows both a global view of translation and the measurement of ribosomal loading and density for specific mRNAs. Simultaneous lysis of infected erythrocytes and parasites releases stable, intact malaria polysomes, which are then purified by centrifugation through a sucrose cushion. The polysomes are resuspended, separated by velocity sedimentation and then fractionated, yielding a characteristic polysome profile reflecting the global level of translational activity in the parasite. RNA isolated from specific fractions can be used to determine the density of ribosomes loaded onto a particular transcript of interest, and is free of host ribosome contamination. Thus, our approach opens translational regulation in malaria to genome-wide analysis.

Keywords

malaria; *Plasmodium falciparum*; polysome; translation; post-transcriptional regulation; global

Beginning with the sequencing of the genome of *Plasmodium falciparum* [1], and continuing with the profiling of both the proteome [2,3] and transcriptome [4,5] global approaches to the study of malaria have led to significant new insights into the biology of

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[§]Co-Corresponding Authors: Jen-Tsan Chi, Duke University Medical Center, Box 3382, 101 Science Drive, CIEMAS 2177A, Durham, NC 27708, Phone: 1-919-668-4759, Fax: 1-919-668-0795. Christopher Nicchitta, Duke University Medical Center, Box 3709, 307 Research Drive, Nanaline Duke Building 436A, Durham, NC 27710, Phone: 1- 919-684-8948, Fax: 1- 919-684-5481.

*These authors contributed equally to this work.

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this parasite. These discoveries include the dynamic stage-specific regulation of malaria transcription across its 48 hour erythrocytic lifecycle [2] and the identification of proteins specific for several host cell stages, including the liver, asexual and sexual erythrocytic stages [6]. These analyses have revealed significant differences in mRNA half-lives across the lifecycle [7], a relative underabundance of annotated transcription factors in the genome [1], and stage-specific upregulation of RNA binding proteins [8], indicating that there is substantial potential for post-transcriptional regulation in the parasite.

Differences between the transcriptomic [4,5] and proteomic [2,3] data highlight translation as a potential key regulatory point for *Plasmodium* gene expression. Indeed, translational regulation is critical for a rapid and reversible response to cell stress and drug exposure [9], and has been implicated in the transition between sexual and asexual stage parasites [10]. In contrast to the genome-wide approaches used to study the malaria proteome and transcriptome, there exist few methods to globally examine translational regulation in *Plasmodium falciparum*.

One of the most common experimental approaches to study translational regulation is polysome profiling. Polysome profiling is used to assess the overall translational activity in a population of cells and to determine the ribosome loading distributions of specific mRNAs of interest. Polysome profiling is achieved by ultracentrifugation of cell extracts, typically in sucrose gradients, to yield the separation of cellular components by hydrodynamic size. The concentration of RNA is determined across the density gradient and provides valuable information about the amount of free ribosomal subunits (40S and 60S, small and large subunits, respectively), monosomes and polysomes present in the sample. By fractionating the gradient, the method can resolve polysomes that differ in size by only a single ribosome, allowing calculation of ribosomal loading and density for a given mRNA [11]. Thus, polysome profiling allows precise studies of translational regulation both at the level of individual genes and across the whole transcriptome [12].

Developing a method for polysome profiling of *P. falciparum* has been met with substantial technical challenges. Previous efforts to purify ribosomes in *Plasmodia* resulted predominantly in the recovery of monosomes, while polysomes were of comparatively low abundance. Electron micrographs of purified ribosomes from *Plasmodium knowlesi* revealed mostly monosomes, although some rare polysomes were seen, composed of 4–6 ribosomes [13]. Polysome profiling of the avian malaria parasite, *Plasmodium lophurae*, similarly revealed a predominance of monosomes with rare polysomes [14]. The only polysome profile obtained for *Plasmodium falciparum* also showed a large monosome peak, but with poor resolution of polysomes and subunits [15]. In total, these earlier approaches were limited in their ability to recover malaria polysomes. Indeed, the difficulties in developing such an approach left it unclear whether substantial populations of polysomes were present in malaria at all. Without a means to purify malaria polysomes, it has not been possible to obtain the global translational profile of the parasite or interrogate the translational regulation of specific mRNAs.

We describe here a novel procedure to determine the polysome profile of *Plasmodium falciparum*. Due to concerns of host cell contamination, several procedures have been tested over time to separate the *Plasmodium* parasite from the host erythrocyte. One of the most common of these is saponin lysis of erythrocytes, followed by purification of the parasites by centrifugation [16]. For reasons that remain unclear, including this initial saponin lysis step in the procedure leads to breakdown of the polysomes into monosomes (Figure 1A). Thus, saponin lysis—which has been used for decades to isolate *Plasmodium* parasites—produces a monosome-dominated polysome profile.

To circumvent this obstacle and obtain the native polysome profile, we directly and simultaneously lysed both the infected erythrocytes and the parasite contained within. We employed a lysis buffer which has been optimized and applied in a variety of experimental contexts for the isolation and profiling of polysomes in eukaryotes [19]. The lysis buffer contains both a high concentration of potassium acetate (400 mM) that is necessary to effectively solubilize membrane-bound ribosomes (thus preventing bias towards cytoplasmic ribosomal fractions), and magnesium (15 mM) to maintain nuclear integrity.

Following simultaneous lysis of the erythrocytes and parasites, we purified the ribosomes and subunits from the lysate by centrifugation through a sucrose cushion. The appearance of the ribosomal pellet varies between runs; it may look transparent and glassy, or may contain reddish-brown insoluble material. (Flash-freezing, storage at -80°C , and thawing of the ribosomal pellet cause no loss to polysomal integrity (data not shown).) The ribosomal pellet was resuspended and loaded onto a continuous sucrose gradient to resolve the ribosomal subunits, monosomes, and polysomes by velocity sedimentation, followed by simultaneous A_{254} detection and fractionation. We have observed that RNA yields vary considerably with the lifecycle stage of the parasite, where trophozoite and schizont stage cultures (20+ hours post-infection) require around half of the input of early ring stage cultures (4–8 hours post-infection). In practice, the minimum input is 60 ml of synchronized infected culture for trophozoites/schizonts versus at least 120 ml for rings at 10% parasitemia.

A typical A_{254} trace is shown in Figure 1B, with the direction of sedimentation from left to right on the graph (lighter fractions to the left). The initial A_{254} is high, likely due to absorbance by hemoglobin in the early fractions. The A_{254} then drops precipitously to baseline. The first peak is small, and corresponds to the small ribosomal subunit (40S). The second peak is slightly taller than the first, and corresponds to the large ribosomal subunit (60S). The next peak is the largest, and corresponds to the monosome peak (80S). Following the monosome peak are the peaks corresponding to polysomes. Each successive peak represents polysomes bearing progressively higher integral numbers of ribosomes (2, 3, 4, etc.). Typical runs allow the resolution of polysomes composed of 5–7 ribosomes, though we have resolved up to 9-mer polysomes in some runs with a larger amount of starting material (~500 μg of total RNA input, equal to 120–180 ml of infected *Plasmodium falciparum* culture at 10% parasitemia and 32+ hours post-infection, and at least 180 ml for earlier infection stages). Higher order polysomes compose the remainder of the trace, and cannot be resolved under these gradient conditions. In order to confirm that the A_{254} signal represents rRNA, qPCR was performed to quantitate 18S and 28S rRNA across the gradient (Figure 1C). The qPCR results corroborate the identities of the A_{254} profile peaks described above.

Concerns are often raised about the contamination of *Plasmodium* samples with nucleated blood cells, and in this case, contamination with host-derived ribosomes. To ensure sample purity, it is advantageous to run uninfected erythrocytes through the above procedure to compare the A_{254} traces of infected and uninfected erythrocytes. When we have done this, we observe no pellet after centrifugation through the sucrose cushion, and see no A_{254} signal above baseline after velocity sedimentation (Figure 1D). Importantly, we also observe that malaria-specific transcripts co-migrate with the polysomal fractions (Figure 2), consistent with the polysomes being parasite-derived.

These studies demonstrate that substantial quantities of polysomes are present in *Plasmodium falciparum* and that the polysomes can be isolated with sufficient quality and purity for translational profiling. With this method, it is now possible to directly examine the global translational status of the parasite. This opens the door for novel investigations into how translation is regulated over the course of the malaria lifecycle and how the

translational profile changes in response to varying experimental conditions. In addition to obtaining a global translational profile, we can apply this approach to investigate the translational activity of specific mRNAs. RNAs can be extracted from the gradient fractions using standard guanidinium thiocyanate-based methods (TRIzol, Invitrogen) or spin columns (RNeasy, Qiagen or *mirVana*, Ambion). The yield depends on the starting amount of parasite, but is generally sufficient for Northern blots, RNA sequencing, microarrays, or qPCR. The A₂₅₄ trace is used to determine the ribosomal loading of mRNAs purified from specific fractions.

As a proof-of-principle, we first sought to demonstrate that our method could distinguish between mRNAs with different ribosomal loading profiles. We compared the sedimentation profiles of four different parasite mRNAs at 32 hours post-infection (Figure 2A). Transcripts for both phosphoethanolamine *N*-methyltransferase (PEAMT, PlasmoDB ID: MAL13P1.214) and knob-associated histidine rich protein 3 (KAHRP, PlasmoDB ID: PfB0100c) co-sedimented predominantly with heavy polysomes. In contrast, a subset (~10%) of mRNAs encoding ring-infected erythrocyte surface antigen (RESA, PlasmoDB ID: PFA0110w) accumulated with the 40S small subunit, suggesting that this subset of RESA transcripts initiates translation with decreased efficiency. A significant subset of cyclic AMP-dependent protein kinase, regulatory subunit (PKA-R, PlasmoDB ID: PfL1110c) mRNAs co-sedimented with 80S monosome (fraction #15), indicating alternative translational regulation of this subset relative to the rest of the PKA-R transcripts. Translational regulation of PKA-R is of particular interest, because this gene is involved in the transition from the asexual to sexual blood stage [17], and translational regulation in the blood stage of *P. falciparum* has previously been demonstrated primarily in gametocytes [10]. We have therefore shown that polysome profiling can be applied in malaria to detect transcript-specific differences in ribosomal loading.

To relate ribosomal loading data to translational regulation, we used analytical methods developed previously by Arava and colleagues [11]. We used the malaria polysome profile to calculate the average number and density of ribosomes loaded onto mRNAs of interest. While the average number of loaded ribosomes increases with ORF length, ribosomal density is of particular interest because it is largely determined by the rates of the three stages of translation: initiation, elongation, and termination. High ribosomal density indicates efficient initiation or inefficient elongation or termination; conversely, low ribosomal density reflects impaired initiation or speedy elongation or termination. Thus, changes in ribosomal density for a specific mRNA point to shifts in translational regulation. Alternatively, extremely high or low ribosomal density can indicate unusual mechanisms of translational regulation specific to that mRNA. (Notably, ribosomal density has been shown in other organisms to decrease as ORF length increases [11]. Until the relationship between ribosomal density and ORF length is established for *P. falciparum* at a genome-wide level, we caution against comparing ribosomal density between different mRNAs, unless the ORFs are of comparable length.)

We applied this analysis to our malaria data as follows: the A₂₅₄ polysome trace (Figure 1B) was used to determine the number of ribosomes per mRNA for each fraction. Using the qPCR data to calculate the percentage of a given mRNA in each fraction (Figure 2), we determined the number of ribosomes loaded per mRNA as a weighted average (Table 1, second column). By obtaining ORF lengths from the most recent assembly of the *P. falciparum* genome (Table 1, third column), we calculated ribosomal density and spacing (Table 1, fourth and fifth columns). Because a eukaryotic ribosome spans ~35 nucleotides [11], we were also able to calculate the percentage of the maximal ribosomal packing density occupied by ribosomes on each transcript (Table 1, last column).

Our initial studies of ribosomal density in *P. falciparum* are consistent with the findings of Arava and colleagues in *Saccharomyces cerevisiae* [11]. For the four genes examined in Figure 2A, we obtained densities ranging from 0.13 to 0.67 ribosomes per 100 nts (Table 1, fourth column), well within the range of 0.03 to 3.3 per 100 nts reported by Arava *et al.* Similar to prior findings in yeast, malaria transcripts do not display maximal ribosome loading (Table 1, final column), suggesting that initiation is rate-limiting under the experimental conditions used. Finally, within our very limited sample we do note a negative relationship between ORF length and ribosomal density, as seen previously. These findings demonstrate that polysome profiling makes it possible to quantitatively analyze translational regulation in malaria, providing a powerful approach to track changes in *Plasmodium* translation at the level of individual genes.

We then applied polysome profiling to examine differences in ribosomal loading and density of a specific mRNA over the course of the *P. falciparum* lifecycle. We chose the RESA gene, which shows marked differences in transcriptional regulation across the 48 hour erythrocytic lifecycle [4]. At 8 hours post-infection (Figure 2B) all of the RESA transcripts were localized in heavy polysome fractions, while at 32 hours post-infection (Figure 2A, 2B) a subset of RESA transcripts co-localized with the small ribosomal subunit, indicative of a decreased efficiency in translation initiation. The average density of ribosomes on RESA transcripts decreased 20% from the 8 to 32 hour time point (Table 1, lower), reflecting the shift in a subset of mRNAs from the polysome pool to the non-translating, small subunit-associated pool. These results demonstrate that polysome profiling is capable of revealing changes in the translation of individual mRNAs throughout the *P. falciparum* lifecycle.

Additionally, proteins can be purified from the fractions by immunoprecipitation or by precipitation with trichloroacetic acid. These approaches are especially useful for studying the protein composition of mRNPs with different ribosomal loading profiles and for studying candidate proteins that modulate translation or act co-translationally.

In summary, we report a robust procedure for the isolation and profiling of polysomes from *Plasmodium falciparum*. With this method, the global translational status of the malaria parasite can be examined at a genome-wide scale, helping to bridge our gap in knowledge between the malaria transcriptome and proteome. Data on the ribosomal loading of transcripts can be readily obtained and analyzed to determine the density of ribosomes on mRNAs for specific genes. Changes in ribosomal density can be used to establish how translation is regulated throughout the parasite lifecycle. We will also be able to screen for genes that show unusual translation profiles, reflecting alternative modes of regulation. Finally, it will be of particular interest to use polysome profiling to evaluate how the malaria translome responds to novel antimalarial agents and to identify translation-based mechanisms of drug resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Isolation of polysomes from *Plasmodium falciparum*. (A) A₂₅₄ profile of polysomes extracted from saponin-lysed *P. falciparum* parasites (synchronized 32 hrs post-infection). (B) A₂₅₄ profile of polysomes extracted by simultaneous lysis of *P. falciparum* in host erythrocytes (synchronized 32 hrs post-infection). (C) qPCR of 18S and 28S *P. falciparum* rRNA from isolated polysomes (n = 4; mean ± s.e.m.) (D) A₂₅₄ profile of uninfected erythrocytes subjected to the polysome purification procedure. *Plasmodium falciparum* (strain 3D7) was cultured under standard conditions [18] using AlbuMAX I as a serum substitute. A minimum of 60 ml of parasite culture at 2% hematocrit and 10% parasitemia is necessary for polysome detection by A₂₅₄. These conditions generate 100–500 µg of total RNA, depending on parasite lifecycle stage. Parasites 4–8 hours post-infection generate 100–150 µg of total RNA, while parasites 32–40 hours post-infection yield ~500 µg total RNA. A thin blood smear Giemsa stain was performed to determine parasitemia. Parasite cultures (with a mixture of infected and uninfected erythrocytes) were treated with 200 µM cycloheximide (CHX) at 37 °C for 10 minutes to arrest elongating ribosomes and stabilize polysomes. The red blood cells (RBCs) at 10% parasitemia were pelleted by centrifugation at 500 *xg* at room temperature, then washed twice with room temperature phosphate-buffered saline (PBS) containing 200 µM CHX. The RBCs were resuspended in PBS with 200 µM CHX, pelleted as above, and kept on ice. The supernatant was aspirated and the pellet was lysed in lysis buffer (1% v/v Igepal CA-630, 0.5% w/v deoxycholate (DOC), 400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM CHX, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 U/ml RNaseOUT (Invitrogen, Carlsbad, CA, USA)) [19] yielding a final lysate volume of approximately 4.25 ml. The lysate was rotated end-over-end at 4 °C for 10 min to achieve complete lysis of the RBCs and parasites, then clarified by centrifugation at 20,000 *xg* at 4 °C for 10 min. To isolate malaria polysomes, 3.75 ml of clarified lysate was layered atop 1.25 ml of 0.5 M sucrose cushion (0.5 M sucrose, 400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM CHX, 1 mM DTT, 1 mM PMSF, 40 U/ml RNaseOUT). Samples were centrifuged for 146 min at 55,000 rpm (4 °C) in an SW-55 Ti rotor (Beckman Coulter). Supernatants were saved, while the ribosomal pellets were flash-frozen in liquid nitrogen and stored at –80 °C for later use. The frozen ribosome pellets were resuspended in 500 µl of ribosome resuspension buffer (400 mM potassium acetate, 25 mM potassium HEPES, pH 7.2, 15 mM magnesium acetate, 200 µM CHX, 1 mM DTT, 1 mM PMSF, 40 U/ml RNaseOUT in RNase-free water) and clarified at 16,000 *xg* at 4 °C for 10 min. The ribosome suspension was layered atop a 10 ml continuous linear 15–50% sucrose gradient (sucrose w/v in ribosome resuspension buffer above), and centrifuged in an SW41 rotor (Beckman Coulter) for 3 hrs at 35,000 rpm (4 °C). Fractions of

330 μ l were collected using an automated Density Gradient Fractionation System (Teledyne Isco, Inc., Lincoln, NE, USA) with a simultaneous A_{254} trace and stored at -80°C . *Symbol legend*: *40S ribosomal subunit, +60S ribosomal subunit, ‡80S monosome.

Fig. 2. Ribosomal loading of *Plasmodium falciparum* mRNAs. (A) Comparison of ribosomal loading profile of several malaria transcripts. Malaria polysomes were harvested, profiled, and fractionated 32 hours post-infection, as described in Fig. 1. Total parasite RNA was isolated from each fraction using a mirVana Isolation Kit (Ambion). Transcript abundance was quantified in fractions across the gradient by SYBR Green qPCR for the following parasite proteins: knob-associated histidine rich protein 3 (KAHRP), ring-infected erythrocyte surface antigen (RESA), cyclic AMP-dependent protein kinase, regulatory subunit (PKA-R) and phosphoethanolamine *N*-methyltransferase (PEAMT) (primers indicated in Supplemental Table 1). Transcript abundance was normalized against total transcript level for each fraction within the gradient ($n = 3$; mean $\pm$ s.e.m.) (B) Lifecycle stage-dependent differences in ribosomal loading. At 8 hours (red) and 32 hours (blue) post-infection, malaria polysomes were harvested, profiled, and fractionated as above. RESA mRNA was quantified in fractions across the gradient by SYBR Green qPCR as described in

(A) (n = 3; mean $\pm$ s.e.m.) *Symbol legend*: *40S ribosomal subunit, +60S ribosomal subunit, ‡80S monosome.

Table 1

Ribosomal loading and ribosomal density of *Plasmodium falciparum* mRNAs.

Gene	Number of ribosomes per mRNA (weighted average) ^b	ORF length (nts) ^c	Number of ribosomes per 100 nts ^d	Ribosome spacing (one ribosome per X nts) ^e	% maximal ribosomal packing density ^f
PEAMT	5.40	801	0.67	148	23.57%
KAHRP	4.02	2312	0.17	575	6.09%
RESA	4.09	3258	0.13	797	4.39%
PKA-R	3.98	1326	0.30	333	10.52%
RESA, 8 hrs p.i. ^a	5.14	3258	0.16	634	5.52%
RESA, 32 hrs p.i.	4.09	3258	0.13	797	4.39%

All calculations adapted from [12].

^a p.i. = post-infection.

^b Sum of (# ribosomes per mRNA for Fraction X) x (% of mRNA in Fraction X) for all fractions.

^c From the *Plasmodium falciparum* (3D7) chromosome sequence and annotation (version: 2010-06-01).

^d (Number of ribosomes per mRNA) ÷ (ORF length) x 100.

^e 100 ÷ (Number of ribosomes per 100 nts).

^f (~35 nts spanned per ribosome) ÷ (ribosome spacing).