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Short technical report

Polysome profiling of the malaria parasite *Plasmodium falciparum*

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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 *P. 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.

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 this parasite. These discoveries include the dynamic stage-specific regulation of malaria transcription across its 48 h 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 *P. 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*,

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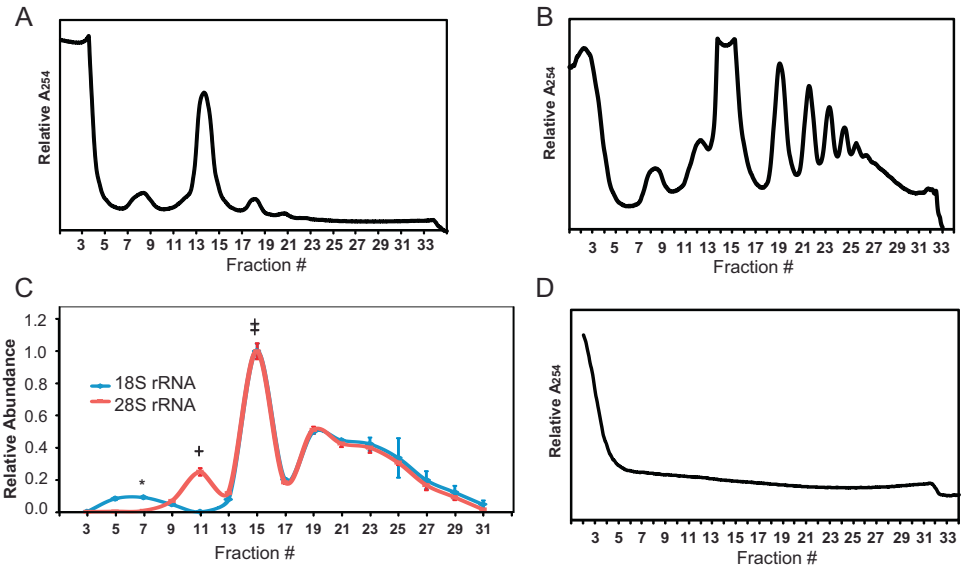

Fig. 1. Isolation of polysomes from *Plasmodium falciparum*. (A) A_{254} profile of polysomes extracted from saponin-lysed *P. falciparum* parasites (synchronized 32 h post-infection). (B) A_{254} profile of polysomes extracted by simultaneous lysis of *P. falciparum* in host erythrocytes (synchronized 32 h post-infection). (C) qPCR of 18S and 28S *P. falciparum* rRNA from isolated polysomes ($n = 4$; mean ± s.e.m.) (D) A_{254} 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 A_{254}. These conditions generate 100–500 μg of total RNA, depending on parasite lifecycle stage. Parasites 4–8 h post-infection generate 100–150 μg of total RNA, while parasites 32–40 h 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 min to arrest elongating ribosomes and stabilize polysomes. The red blood cells (RBCs) at 10% parasitemia were pelleted by centrifugation at 500 × *g* 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 phenylmethylsulfonate 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 × *g* 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 × *g* 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 h at 35,000 rpm (4 °C). Fractions of 330 μl 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, and ‡ 80S monosome.

similarly revealed a predominance of monosomes with rare polysomes [14]. The only polysome profile obtained for *P. 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 *P. 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 (Fig. 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 trophozite and schizont stage cultures (20+ h post-infection) require around half of the input of early ring stage cultures (4–8 h 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 Fig. 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