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Two long non-coding RNAs generated from subtelomeric regions accumulate in a novel perinuclear compartment in *Plasmodium falciparum*

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Abstract

Chromosome ends have been implicated in the default silencing of clonally variant gene families in the human malaria parasite *Plasmodium falciparum*. These chromosome regions are organized into heterochromatin, as defined by the presence of a repressive histone H3 lysine 9 trimethylated marker and heterochromatin protein 1. Here, we show that the non-coding subtelomeric region adjacent to virulence genes forms facultative heterochromatin in a cell cycle-dependent manner. We demonstrate that telomere-associated repeat elements (TAREs) and telomeres are transcribed as long non-coding RNAs (lncRNAs) during schizogony. Northern blot assays revealed two classes of lncRNAs: a ~4-kb transcript composed of telomere sequences and a TARE-3 element, and a >6-kb transcript composed of 21-bp repeats from TARE-6. These lncRNAs are transcribed by RNA polymerase II as single-stranded molecules. RNA-FISH analysis showed that these lncRNAs form several nuclear foci during the schizont stage, whereas in the ring stage, they are located in a single perinuclear compartment that does not co-localize with any known nuclear subcompartment. Furthermore, the TARE-6 lncRNA is predicted to form a stable and repetitive hairpin structure that is able to bind histones. Consequently, the characterization of the molecular interactions of these lncRNAs with nuclear proteins may reveal novel modes of gene regulation and nuclear function in *P. falciparum*.

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Author contribution

R.H.-R designed research; M.S.-M. and D.M.D performed research, L.M-S and M.V. help in the RNA-FISH assays, S.M-C help in the run-on assays, N.V-S performed RNA secondary structure, A.S and R.H.-R. analyzed data and wrote the paper.

Conflict of interest statement

The authors declare no conflict of interest.

Keywords

Long ncRNA; Nuclear compartment; Nuclear periphery; *Plasmodium falciparum*; RNA–protein complex; Subtelomere

1 Introduction

In recent years, heterochromatin at chromosome ends has been the focus of many studies because it plays an important role in regulating gene expression in both yeast and humans [1,2]. Similarly, in the protozoan *Plasmodium falciparum*, which is the most virulent human malaria parasite, subtelomeres exert a silencing effect on the clonally variant virulence gene families. The chromosome ends in this pathogen are organized into heterochromatin, as defined by the presence of high levels of the repressive histone H3 lysine 9 tri-methylation (H3K9me3) [3,4] and heterochromatin protein 1 (PfHP-1) [5,6]. This heterochromatin is associated with a 1.2-kb non-coding telomere repeat region (GGGTTT/CA) and an adjacent non-coding region that together form a mosaic of six different blocks of repetitive sequences known as telomere-associated repetitive elements (TAREs) 1–6 [7]. Adjacent to TARE-6 are members of several gene families that encode variant surface proteins, such as *var*, *rif*, *stevor* and *pfmc-2tm*. These gene families are, by default, epigenetically repressed and undergo phenotypic variation through *in vitro* switches. Expression in a single cell occurs either by mono-allelic expression (*var* gene members) or by co-transcription of several members (*stevor*, *rif* and *pfmc-2tm*) [7,8]. The overall chromatin and DNA organization of the chromosome ends is highly conserved among all the chromosomes of the malaria parasite [3,7,8]. In addition to the identified heterochromatin factors H3K9me3 and PfHP-1, a number of histone-modifying enzymes and other factors with unknown functions, such as PfSir2, PfOrc1 and PfKMT1, are recruited to subtelomeric regions [3,6,9]. Experimental evidence suggests that interactions between these proteins and the telomeric and subtelomeric regions generate a repressive epigenetic center [3,10]. This repressive center may extend to adjacent virulence gene families to create a molecular platform for the phenotypic variation of virulence factors.

Specialized nuclear compartments are crucial for the function of the *Plasmodium* nucleus [11], and they are particularly relevant for the mutually exclusive expression of *var* genes when an active *var* gene is placed in the *var* expression site. However, the model that describes this expression site as the limiting factor for the expression of a single *var* gene needs to be reconsidered because this expression site can harbor more than one transcriptionally active *var* gene [12]. Finally, fluorescence *in situ* hybridization assays with DNA (DNA-FISH) have shown that an active episomal *rifin* promoter colocalizes with a transcriptionally active *var* promoter, indicating that these gene families utilize the same subnuclear expression site [13]. Therefore, specialized nuclear compartments are important for the regulation of expression of several multi-copy gene families and for rDNA in distinct perinuclear compartments [3,10,14,15].

Although multiple epigenetic factors contribute to the coordinated expression of virulence genes, the role of non-coding RNAs (ncRNAs) remains, for the most part, unexplored in *P.*

falciparum. Initial evidence indicating a role for ncRNAs in malaria parasites was provided by Kyes et al. in 2003, who described *var*-associated ncRNAs [16]. Later, Deitsch and colleagues identified non-coding transcripts in *P. falciparum* from centromeric repeats [17]. In addition, the same group showed that an intron-derived ncRNA is enriched in the chromatin of *var* genes, but no functional data were presented [18]. More recently, a genome-wide study in *P. falciparum* revealed the presence of short transcripts derived from non-coding subtelomeric regions [19,20], suggesting that the heterochromatic chromosome ends can be transcribed. A more recent study using microarray analysis suggested that long ncRNA (lncRNA) are produced from TARE-3 repeats [21].

In the present study, we used northern blot, FISH and run-on analyses to demonstrate that *P. falciparum* telomeres and non-coding subtelomeric repeat regions are transcribed as heterogeneous long ncRNAs (lncRNAs) by RNA polymerase II. We found that only the sense strand is transcribed in asexual blood stages. Transcription peaks occur during the schizont stage and produce two lncRNAs: a ~4 kb lncRNA that contains TARE-3 and telomere sequences (lncTARE-3-Telomere) and a >6 kb lncRNA that consists of 21-bp repeats from TARE-6 (lncTARE-6). After transcription, both lncRNAs relocate to a single novel nuclear compartment that is distinct from the chromosome ends. Furthermore, the data from gel shift, super gel shift and northwestern blot assays suggest that this lncRNA may form large networks of interlinked RNA–protein complexes whose functions are unknown.

2 Materials and methods

2.1 Parasites

The *P. falciparum* strain FCR3 was maintained according to standard culture conditions [22]. Panning assays for the selection of FCR3 parasites that transcribe *var* genes associated with chondroitin-sulfate A (CSA) binding were performed as previously described [23].

2.2 Nuclear and total RNA purification and northern blotting

Nuclear RNA was prepared from asynchronous cultures of FCR3 *P. falciparum*. Parasites were isolated from infected erythrocytes by saponin lysis, resuspended in 1 ml of lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.65% NP-40) and incubated for 30 min at 4°C. The parasites were then lysed by 200 strokes with a prechilled Dounce homogenizer, and the nuclei were collected by centrifugation. Nuclear and total RNA were purified using TRIzol Reagent (Invitrogen) and solubilized in deionized formamide as described previously [24]. For northern blot analysis, 8 µg of nuclear RNA was treated with RNase-free DNase (Ambion), size-fractionated on a 1.2% agarose gel, supplemented with 5 mM guanidine thiocyanate and transferred to a Hybond N+ nylon membrane (Amersham). The membranes were prehybridized for 3 h at 42°C in 5X SSC, 50% formamide, 5X Denhardt's solution, 1% SDS and 100 µg of herring sperm DNA and hybridized overnight at 42°C in 5X SSC, 50% formamide, 5X Denhardt's solution, 1% SDS, and 5% dextran sulfate with α -³²P[dATP]-labeled probes (Megaprime, GE Healthcare). The filters were washed three times for 30 min each with 0.2X SSC/0.1% SDS at 42 °C, and the film was developed after exposure to an intensifying screen at –80°C. Probes for the telomere, TARE-3 and TARE-6 regions were obtained as described elsewhere [9].

2.3 Dot-blot analysis

Dot-blot assays were performed as previously described [9]. Briefly, DNA or RNA samples were placed on a nylon membrane (Hybond N+). For RNA probes, the membrane was prehybridized in a solution containing 5X SSC, 50% formamide, 5X Denhardt's solution, 1% SDS, and 100 µg herring sperm DNA for 3 h at 50°C. Hybridization was performed in the same solution for 16 h at 50 °C, the filters were washed three times with 2X SSC/0.1% SDS at 50°C, and the film was developed after exposure to an intensifying screen at -80 °C.

2.4 Molecular cloning into M13 and preparation of single-stranded DNA

DNA fragments from the telomere, TARE-3 and TARE-6 regions were obtained after the digestion of previously obtained plasmids [9] and cloned into M13mp18 and M13mp19 replicative-form DNA [25]. XL1-Blue competent cells were transformed with a cloning mix, and single-stranded DNA was purified from colorless plaques using QIAprep Spin M13 columns (QIAGEN) as specified by the supplier.

2.5 Nuclear run-on assays

Nuclear run-on assays were performed as previously described [26]. Cells were washed once in 1X PBS, then gently lysed with saponin to release parasites from red blood cells (RBCs) and to reduce the bulk of uninfected RBCs (0.005% saponin in 1X PBS, 6 ml per 0.5 ml of packed infected RBCs, on ice for 5 min), followed by centrifugation for 5 min at 2000 rpm (4°C). The resulting pellets were pooled in 3 ml of 1X hypotonic lysis buffer (10 mM Tris-Cl, pH 7.5, 10 mM NaCl, 2.5 mM MgCl₂, 14 mM β-mercaptoethanol).

NP40 (0.5%) was then added to release the nuclei from the parasites, the samples were centrifuged for 5 min to pellet the nuclei (12,000 rpm for rings, 5000 rpm for trophozoites at 4 °C), and the resulting pellet was washed once in 1X hypotonic lysis buffer to remove residual NP40. The nuclei were then resuspended in 100 µl of 2X transcription buffer (40 mM Tris-HCl, pH 7.5, 140 mM KCl, 10 mM MgCl₂, 40% glycerol), and the final volume was adjusted to 200 µl with RNase-free water. One microliter of 10 mg/ml α-amanitin was added (final concentration of 0.1 mg/ml) to half of the nuclei in transcription buffer (100 µl), and water was added to the remaining control nuclear sample. The nuclei, with or without the inhibitor, were equilibrated for 15 min on ice, and the following reagents were then added from 0.1 M stocks at the indicated final concentrations: 1 mM DTT, 4 mM ATP, 1 mM GTP and 1 mM CTP. To each 107 µl of reaction solution, 2.5 µl of α³²P-UTP (3000 Ci mmol⁻¹; 10 mCi⁻¹; final ~0.08 mM; PerkinElmer) was added, and the reactions were equilibrated for 3 min on ice. The reactions continued for 5 min at 30 °C, and non-radioactive UTP was added at a final concentration of 5 mM to each tube, followed by an additional 10-min incubation at 30 °C. The transcription reaction was stopped by the addition of 1 ml of TRIzol with 1 µl of 10 mg/ml yeast tRNA as a carrier (Sigma). The labeled nascent RNA and total RNA were then processed as previously described [24]. Briefly, 0.2 ml of chloroform was added, followed by a 13,000 rpm spin for 25 min at 4 °C. The aqueous layer (0.6 ml) was precipitated in 0.5 ml of isopropanol on ice for 1–2 h, vortexed and centrifuged for 30 min at 4 °C and 13,000 rpm. The pellet was resuspended and denatured by adding 100 µl of formamide, heated to 65 °C for 5 min, centrifuged for 1 min at 4 °C and then placed on ice until it was added to 3 ml of hybridization solution (7%

sodium dodecyl sulfate/0.5 M sodium phosphate buffer pH 7.2, 2% dextran sulfate and 1 mM EDTA) [24]. The hybridization solution containing the probe was added to a prehybridized Southern blot filter and incubated at 50°C overnight. The blots were washed in 0.2X SSC (0.3 M NaCl, 3 mM Na citrate, pH 7/0.1% SDS) at 50–55 °C.

2.6 In vitro transcription of telomeric, TARE-3 and TARE-6 regions

Plasmids containing telomeric, TARE-3 and TARE-6 fragments were used as templates for *in vitro* transcription. Three amplicons, a 170-bp amplicon from the telomere, a 569-bp amplicon from TARE-3 and a 70-bp amplicon from TARE-6, were produced by PCR using the following primers: sense Telomere/T7: 5'-TAATACGACTCACTATAGGGAATCCGTCGAGCAGCAGGTGTTT-3'; antisense Telomere/T3: 5'-TCCCTTTAGTGAGGGCAGAATTCGGCTTGAATTCC-3'; sense TARE3/T7: 5'-TAATACGACTCACTATAGGG GGAAAAATGGTGTATTGTTAC-3'; antisense TARE3/T3: 5'-TAATACGACTCACTATAGGGTATGAATGAAAAATGGGTTG-3'; sense TARE6/T7: 5'-TAATACGACTCACTATAGGGTAGGTTTTAGGTAAAAGCAAC-3'; antisense TARE6/T3: 5'-TAATACGACTCACTATAGGGTA TACCACACATGTTGGTTAT-3'. The PCR sense and antisense primers contained bacteriophage T7 and T3 promoter sequences (underlined nucleotides), respectively, and an additional GG to enhance transcription. Purified PCR products were used as templates for RNA synthesis using an *in vitro* transcription kit (MEGAscript T7, Ambion) according to the manufacturer's protocol. DNA templates and unincorporated nucleotides were removed by DNase I (Ambion) treatment in the presence of RNase inhibitors (Sigma) and by gel filtration, respectively. For the synthesis of labeled RNA transcripts, biotin-16-UTP (Ambion) was included in the transcription reaction.

2.7 Fluorescence in situ hybridization

RNA-FISH and combined RNA/DNA-FISH were performed as previously described [3]. DNA probes were obtained as previously described [15]. RNA probes were labeled by *in vitro* transcription in the presence of biotin-16-UTP or Alexa Fluor 488-5-UTP. For RNA-FISH, infected (RBC) were lysed with saponin, and the released parasites were fixed in suspension with 4% paraformaldehyde in RNase-free conditions. The parasites were then deposited on microscope slides, treated with 0.1% Triton X-100 for 5 min and hybridized with single-stranded RNA probes at 37 °C for 16 h. After washing, the slides were incubated with streptavidin conjugated with Alexa Fluor 568 (1:1000 in PBS/4% BSA) at room temperature for 30 min and washed in PBS/0.5% Tween 20. The slides were then washed with 2X SSC at 37°C and mounted with Vectashield containing DAPI to detect biotin RNA probes. For sequential RNA/DNA-FISH experiments, the parasites were first hybridized with RNA probes at 37 °C as described previously, then washed, denatured at 80°C for 30 min and hybridized with DNA probes (telomere and 28S rRNA) at 37 °C for 16 h. Finally, the slides were washed in 2X SSC/50% formamide for 30 min at 37 °C followed by subsequent washes in 1X SSC, 2X SSC and 4X SSC for 10 min each at 50 °C prior to mounting.

2.8 In vitro secondary structure prediction

The secondary structure of the TARE-6 long non-coding RNA was predicted with RNAfold software [27] using the Vienna RNA server [28].

2.9 Northwestern blot and EMSA

Northwestern blotting assays were carried out as described by Mears and Rice [29] with some modifications. Nuclear extract proteins were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane followed by three washing steps with northwestern (NW) buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1X Denhardt's solution and 2 mM DTT). The transferred proteins were then renatured *in situ* for 12 h at 4 °C in NW buffer. The membranes were incubated at room temperature (20–25 °C) for 2 h in NW buffer containing 100 µg/ml yeast tRNA. Following the addition of 5×10^6 c.p.m. of a ^{32}P -labeled RNA probe, the proteins were incubated for an additional 6 h. Then, the membranes were washed three times in NW buffer (10 min each) at room temperature and subsequently analyzed by autoradiography. For RNA electrophoretic mobility shift assays, the purified proteins were incubated with 5×10^4 c.p.m. of a 70 nt ^{32}P -labeled RNA probe for 30 min at 4 °C in binding buffer (10 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM DTT and 1% Triton X-100). The RNA probe was obtained by *in vitro* transcription as described previously. The RNA-protein complexes were then analyzed by electrophoresis on a 6% native polyacrylamide gel followed by autoradiography. The amount of protein was normalized using Bradford protocol.

2.10 Histone extraction

The histones from *P. falciparum* were obtained using the protocol described by Longhurst in 1997 with some modifications [30]. Briefly, the *P. falciparum* strain FCR3 parasites were isolated from infected erythrocytes by saponin lysis. The pellet was resuspended in 1 ml of lysis buffer (10 mM Tris HCl, pH 8, 1.5 mM MgCl_2 , 1.5 mM KCl, 0.25% NP-40) and incubated for 30 min at 4°C. The parasites were then lysed by 200 strokes with a prechilled Dounce homogenizer, and the nuclei were collected by centrifugation and washed once with 10 volumes of 0.3 M NaCl and twice with 0.5 M NaCl. Then, the histones were extracted by incubating the samples overnight at 4 °C with 10 volumes of 0.25 M HCl. The extracts were centrifuged for 30 min to remove insoluble debris. The supernatant was precipitated at 4 °C with eight volumes of cold acetone and centrifuged for 30 min. The pellets were dried, resuspended in Tris-HCl pH 8.8 and stored at –80°C.

3 Results

3.1 Telomeric and TARE regions are transcribed as sense RNAs by RNA polymerase II

To determine whether the chromosome end regions of *P. falciparum* are transcribed as long non-coding RNAs, we performed run-on assays. The resulting *de novo* RNAs were hybridized with positive and negative single-stranded M13 DNA probes from the telomere and the TARE-3 and TARE-6 regions. The polarity of the single-stranded DNA was established with respect to the subtelomeric *var* gene (PFI1830c) located on chromosome 9 (Fig. 1A). A hybridization signal was observed with three negative single-stranded probes,

whereas no signal was detected with the positive single-stranded probes (Fig. 1B). To verify these results, RNA-FISH assays were performed using biotin-16-UTP-labeled sense and antisense transcripts of the telomeric, TARE-3 and TARE-6 regions. A fluorescent signal was observed at the periphery of the nucleus and, as expected, only when parasites were incubated with the antisense RNA probes. No signal was observed with the sense transcripts (Suppl. Fig. 1). Collectively, these data indicate that the telomeric and TARE regions are transcribed only as sense transcripts.

To identify the RNA polymerase responsible for transcribing the telomeric and subtelomeric regions in *P. falciparum*, nuclear run-on assays were performed in the presence or absence of the RNA polymerase II inhibitor α -amanitin. Following treatment, the nascent RNA was collected and incubated with membranes containing DNA from the telomeric region, TARE-3, TARE-6 and the DBL1 domain of the *var1CSA* gene (positive control), which is transcribed by RNA polymerase II [31]. The presence of α -amanitin completely abolished the signal for each of the four regions, whereas the 28S rRNA control (transcribed by RNA polymerase I) remained unaffected, indicating that RNA polymerase II transcribes the telomeric and subtelomeric regions (Fig. 1C).

3.2 The telomeric and TARE regions of *P. falciparum* are transcribed as very large ncRNA transcripts

Telomeric and subtelomeric transcripts are enriched in the nuclear fraction of humans and mice [32,33]. To determine whether the telomeric and TARE transcripts are also enriched in the nuclear fraction of *P. falciparum*, dot-blot assays were performed on total and nuclear RNA from asynchronously parasites. A more intense signal was observed for nuclear RNA compared with total RNA following the hybridization of the telomeric and subtelomeric (TARE-3 and TARE-6) probes with equivalent amounts of RNA (Suppl. Fig. 2). These data indicate that in *P. falciparum*, the telomeric, TARE-3 and TARE-6 transcripts are enriched in the nuclear RNA fraction. Therefore, all subsequent assays were performed using nuclear RNA. To verify that hybridization occurred specifically with the RNA, the same samples were incubated with RNase A prior to blotting. As expected, the hybridization signals disappeared, confirming that the telomere and the subtelomeric TARE-3 and TARE-6 regions are transcribed (Suppl. Fig. 3).

To assess whether the expression of these transcripts is stage-specific in *P. falciparum*, dot blot assays were performed using nuclear RNA from each stage of the 48-h *P. falciparum* asexual blood-stage cycle (0–16 h, ring; 16–36 h, trophozoite; 36–48 h, schizont). The signal was observed in the schizont and ring stages, but the hybridization almost disappeared in the trophozoite stage (Fig. 2A), indicating that these transcripts are expressed in the schizont and ring stages. As a control, we probed a gene (seryl tRNA synthetase) known to be constitutively transcribed throughout the cycle.

To determine the sizes of these transcripts and to confirm that they are expressed in a stage-specific manner, northern blotting was performed using nuclear RNA obtained from each stage. Hybridization with the telomeric probe revealed a smear from approximately 100 bp to at least 6 kb (Fig. 2B). Because the average size of the telomeric repeats is approximately 1.2 kb, the larger transcripts were likely to contain subtelomeric regions as well. To test this

hypothesis, the nuclear RNA was hybridized with probes derived from the subtelomeric region (TARE-3 and TARE-6 elements). Upon hybridization of the nuclear RNA with probes derived from the TARE-3 region, a telomere-like hybridization pattern was generated (Fig. 2B). The TARE-3 region has an average length of approximately 2.1 to 2.8 kb, TARE-2 is around 1.6 kb, TARE-1 is 0.9 kb, and the telomere is 1.2 kb [7]. Thus, the data suggest that the non-coding TARE-3 transcripts are composed of the following telomeric and subtelomeric regions: TARE1, TARE2 and TARE3 (lncTARE3-Telomere).

Surprisingly, when we performed hybridization with the TARE-6 probe, we observed large bands of more than 10 kb (Fig. 2B). Because the average size of this region is between 8.4 and 21 kb [7], it is possible that this transcript is composed of only TARE-6 elements. Taken together, these results suggest that the telomeric and subtelomeric regions of *P. falciparum* are transcribed to generate at least two long ncRNAs, one composed of telomeric and subtelomeric regions (TARE-1, TARE-2 and TARE-3) and the other consisting of the highly repetitive and polymorphic 21-bp repeats (Fig. 2B).

The smear observed with the three probes in the northern blot experiments, could reflect either the processing of this transcript or degradation of the RNA. To rule out the latter possibility, we randomly chose one of the membranes to be washed and re-hybridized with an 18S rRNA probe. As shown in Fig. 2B, a 2.5 kb band representing the 18S probe was detected, indicating that the RNA was intact. Therefore, it is likely that the processing of the long ncRNA into small molecules produced the smear. Moreover, longest lncRNA transcript signals were observed with the three probes in the schizont stage as compared to all other stages (Fig. 2B). However, the smear signal was more evident in the ring stage, indicating that the processing of both lncRNAs may start in this stage and the signal almost disappeared during the trophozoite stage, suggesting that the lncRNAs are completely processed in this stage (Fig. 2B).

3.3 Long non-coding subtelomeric RNAs accumulate in a novel perinuclear compartment in *P. falciparum*

To investigate whether the telomeric, TARE-3 and TARE-6 transcripts co-localize or are targeted to different sites within the nucleus, we assessed their localization at different stages using RNA-FISH. TARE-6 ncRNA was visualized as nuclear foci that colocalized with TARE-3 and telomere repeats during the schizont (early and late) stage and ring stage (Fig. 3A and B). No signal was observed at the trophozoite stage. These foci were located at the nuclear periphery. In the ring stage, a single perinuclear focus was observed.

The nucleus of *P. falciparum* contains various subcellular subdomains, such as telomeric clusters, the perinuclear site of *var* gene expression, and the nucleolus. The presence of large transcripts from the telomeric and subtelomeric regions in the nuclear periphery suggests that they are involved in the formation of telomeric heterochromatin [32,33], and as a result, they may colocalize with telomeric clusters. To investigate this hypothesis, the transcript for TARE-6 ncRNA (obtained by *in vitro* transcription and labeled with biotin-16-UTP) was used as a probe and incubated with paraformaldehyde-fixed parasites to perform RNA-FISH assays. The samples were then hybridized *in situ* with a TARE-6 DNA probe. This sequential RNA/DNA-FISH showed that the TARE-6 lncRNA did not co-localize in most

cases with the subtelomeric DNA clusters during the schizont stage (Fig. 4A). This implies that only a restricted number of subtelomeres are actively transcribed. Alternatively, the transcription of this non-coding RNA could be an event that occurs only for a short time, with transcripts rapidly organizing into a new nuclear compartment. In the ring stage, the lncRNA foci were clearly dissociated from the telomere clusters (Fig. 4A). Next, we determined whether the ncRNAs are associated with other nuclear compartments, such as the nucleolus and the transcriptional activation site of a single *var* gene. A schematic of the known nuclear compartments of *P. falciparum* is shown in Fig. 4B. We performed RNA/DNA-FISH assays in which the TARE-6 non-coding RNA was analyzed by RNA-FISH and the 28S rDNA was subsequently detected by DNA-FISH to assess the possibility that this telomeric non-coding RNA forms part of the nucleolus. Both probes hybridized with the parasites, but their localizations did not overlap, demonstrating that the non-coding RNA compartment is distinct from the nucleolus (Fig. 4C). To determine whether the ncRNAs are associated with the transcriptional activation site of a single *var* gene, which is also known to localize to the nuclear periphery [3,14], we performed RNA-FISH assays in a strain of parasites in which the *var2CSA* gene is actively transcribed (FCR3-CSA). The parasite populations were hybridized with both a *var2CSA* antisense RNA probe and a TARE-6 non-coding RNA probe. No co-localization was observed between the probes, suggesting that the TARE-6 noncoding RNA is not associated with the *var* gene expression site (Fig. 4D). Thus, these data strongly suggest that the ncRNAs define a novel nuclear subcompartment in *P. falciparum*.

3.4 TARE-6 ncRNA 21-bp repeats form a complex hairpin structure and bind nuclear proteins

Considering that certain long non-coding RNAs serve as structural docking sites for the nucleation of specific nuclear proteins in humans [34], the putative secondary structure of the TARE-6 transcripts was investigated. Interestingly, when 1000 bases of the TARE-6 region were used to obtain an *in silico* secondary structure, a stable, complex structure composed of approximately 12 differentially sized hairpin-loop structures and with a minimum free energy of -356 kcal/mol was observed (Fig. 5A). The *in silico* analysis also predicted the formation of hairpin-loop structures by RNA segments containing 2, 3, 4 or 5 repetitive 21-bp repeat elements with free energies of -13 , -20 , -30 and -37 kcal/mol, respectively (Fig. 5B and data not shown). Therefore, various stable stem-loop structures consisting of two to five 21-bp repeats can form alongside this RNA. Taken together, these data suggest that the TARE-6 lncRNA is able to form a stable and complex secondary structure that could serve as a platform for interactions with numerous nuclear proteins.

To determine whether the TARE-6 non-coding RNA binds nuclear proteins, RNA gel shift assays were performed. As shown in Fig. 6, when the TARE-6 ncRNA was incubated with *P. falciparum* nuclear extracts, three well-defined RNA-protein complexes, denoted C1, C2 and C3, were observed (Fig. 6A). The specificity of these complexes was confirmed using a 100-fold molar excess of either a homologous or heterologous competitor. The unlabeled homologous competitor (cold TARE-6 ncRNA) disrupted the formation of all three complexes, whereas the same amount of scrambled probe (heterologous competitor) did not alter the formation of the complexes (Fig. 6A). Collectively, these data suggest that TARE-6

ncRNA binds specifically to nuclear proteins. The interaction of nuclear proteins was also verified by northwestern blotting assays. For this purpose, nuclear and cytoplasmic extracts from *P. falciparum*, total extracts from *Escherichia coli* and a GST protein were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, re-natured *in situ* and incubated with ³²P-labeled TARE-6 ncRNA, which was generated by *in vitro* transcription. As shown in Fig. 6B, the TARE-6 ncRNA bound to three bands with molecular weights between 13 and 17 kDa in the nuclear extract of *P. falciparum* (Fig. 6B). In contrast, no signal was observed in the cytoplasmic parasitic extract, in the total *E. coli* extract or with the GST protein (Fig. 6B). As a negative control, the same assay was performed using a scrambled ³²P-labeled RNA, and as expected, we did not detect any signal (data not shown). Taken together, these data suggest that the TARE-6 ncRNA is able to bind directly to small nuclear proteins in a specific manner.

Next, northwestern assays were performed to determine whether the small nuclear proteins detected in the *P. falciparum* nuclear extracts correspond to histones. The TARE-6 RNA probe hybridized with two bands with approximate molecular weights of 14 to 16 kDa in the *P. falciparum* histone preparation and with one protein band in the calf histone sample (Fig. 6C). As expected, no signal was observed with the GST protein alone (Fig. 6C). On the basis of their sizes, the bands were predicted to correspond to histones H3, H2A and H2B. To determine whether histone H3 binds to TARE-6 lncRNA, EMSAs were performed in the presence of an anti-H3 antibody. As shown in Fig. 6A, three well-defined complexes were observed when the TARE-6 RNA probe was incubated with the *P. falciparum* nuclear extracts (Fig. 6D). However, when an anti-H3 antibody was incubated with the *P. falciparum* extract prior to the addition of the RNA probe, the formation of complex 1 (C1) was abolished, and the presence of a super complex was observed (Fig. 6D). The same amount of control anti-GST antibody did not alter the formation of the three complexes (Fig. 6D). Taken together, these data show the specific interaction of TARE-6 transcripts with histone H3.

4 Discussion

In this work, we characterized a novel class of long non-coding RNAs that are transcribed from the repetitive non-coding subtelomeric and telomeric regions of *P. falciparum* chromosomes. We also showed that these long non-coding RNAs have several potentially interesting properties, including RNA polymerase II-mediated transcription, recruitment to a novel perinuclear subcompartment and specific binding to histone H3.

The northern blot results suggest that multiple transcription start sites produce a variety of lncRNAs from the subtelomeric region (Fig. 2). It remains unclear whether specific promoters are used or if multiple start sites exist within the repeated region, but our results suggest that there are at least two different long non-coding RNAs. The transcription of the first (lncTARE-3-Telomere) appears to start in the TARE-3 region, and it continues until the telomeric region, as was recently reported [21]. The other lncRNA (lncTARE-6), which is 8.4 to 21 kb in length, appears to contain only TARE-6 (21-bp repeats). This element varies in length at different chromosome ends (6 to 23 kb) [7]. The size variation of the lncRNA indicates that distinct chromosome ends are transcribed in bulk culture. The smears observed

in IncTARE3-Telomere and IncTARE-6 suggest that these lncRNAs are generated in the schizont stage (Fig. 2) and are then processed into shorter and perhaps more stable ncRNAs in the ring stage. Nuclear exosomes, which contain 3'-to-5' exonucleases, may participate in RNA processing and quality control [35]. To date, exosomes have not been characterized in this parasite. Because chromosome end breakage and healing are common in *P. falciparum* [36], we cannot exclude the possibility that telomeric lncRNAs are also produced from the end regions of truncated *P. falciparum* chromosomes, which can be three times longer than average (1.2 kb) [37]. In this scenario, the longer telomeric transcripts could be produced by the promoter regions of genes that have moved adjacent to the repaired chromosome ends.

In ring-stage *P. falciparum* parasites, chromosome ends are organized into constitutive heterochromatin [3]. However, the transcription of telomeric and subtelomeric regions from *P. falciparum* strongly suggests that TAREs and telomeres temporarily reorganize their heterochromatin at the end of the blood stage to allow for RNA polymerase II-mediated transcription. Consistent with this model, the chromosome ends are not clustered during cell division (early schizont stage) [9], which may increase their accessibility to the transcriptional machinery. The question of how many chromosomes reorganize into facultative heterochromatin is important. Northwestern and super-shift gel results indicated that TARE-6 lncRNA is able to bind histones (Fig. 6C and D), thereby we speculated that this lncRNA could be functioning as a histone chaperone (probably helping in the assembly and/or disassembly of subtelomeric heterochromatin) during the transcription of chromosome ends of *P. falciparum*.

Our RNA/DNA-FISH data from schizonts (multinuclear cell) suggest that only a few telomere clusters co-localize with the ncRNAs, indicating that they may be generated from one or only a few chromosome ends and that qualitative differences may exist between subtelomeric regions. Given the similarity in organization and sequence composition of the *P. falciparum* chromosome ends, it will be challenging to address this question experimentally.

Our work demonstrates that DNA-dependent RNA polymerase II plays a major role in the transcription of long non-coding telomeric and subtelomeric RNAs because the treatment of *P. falciparum* parasites with the specific RNA polymerase II inhibitor α -amanitin completely abrogated the transcription of telomeres and TAREs (Fig. 1C). In contrast, in both humans and mice, some telomeric transcripts (called TERRA, Telomeric Repeat-containing RNA) are still detectable after prolonged α -amanitin treatment, suggesting that polymerases other than RNA polymerase II may participate in telomere transcription [32,33]. In addition, FISH assays have demonstrated that TERRA preferentially localizes to telomeres and participates in the formation and maintenance of telomeric heterochromatin [33]. However, our FISH data indicate that long ncRNAs are localized in the nuclear periphery and do not colocalize with a TARE-6 DNA probe in ring-stage parasites. These data indicate that in *P. falciparum*, long non-coding telomeric and subtelomeric RNAs may have a different function than in humans.

The nucleus of *P. falciparum* has a complex three-dimensional organization consisting of various specialized perinuclear subdomains, such as telomeric clusters, perinuclear

heterochromatin, *var* gene expression sites and the rDNA cluster that forms the nucleolus [11,15,38]. However, our RNA-FISH and RNA/DNA-FISH results establish that the long ncRNAs from the telomeric and subtelomeric regions do not localize to any of the known subcompartments. Therefore, this work defines a novel perinuclear compartment in *P. falciparum*. Long non-coding RNAs can function as scaffolds for nuclear bodies in other eukaryotes [39]. These nuclear bodies are membraneless sub-organelles, some of which, termed paraspeckles, use long ncRNAs as platforms for their assembly [40]. Further studies are necessary to explore whether the telomeric and subtelomeric long ncRNAs of *P. falciparum* are involved in the assembly of a new nuclear compartment.

Certain biological activities of long ncRNAs are mediated through the creation of a protein-binding module *via* their secondary and tertiary structures [34,41]. In *P. falciparum*, TARE-6 is the largest repetitive element (6 to 23 kb) of the subtelomeric region [7,8,41]. TARE-6 consists of large arrays of degenerate 21-bp repeats. The predicted secondary structure of the TARE-6 lncRNA (Fig. 5) implies that the repeats interact with each other to form long, irregular, repeated stem-loop structures. Thus, TARE-6 lncRNA may form a scaffold that binds proteins and brings them to specific genomic *loci*, thereby allowing them to regulate chromatin structure over a single promoter, a gene cluster or an entire chromosome, as seen in other organisms [42–44]. In this report, we utilized northwestern blotting and EMSA to demonstrate the ability of three 21-nt RNA repeats of TARE-6 to interact with nuclear proteins. Then we proposed that the large TARE-6 lncRNA may form extensive networks of interlinked RNA–protein complexes that may create a specific nuclear environment that could participate in specific tasks.

A major function of long ncRNAs appears to be modulating the epigenetic status of specific proximal and distal genes in eukaryotes. For example, *HOTAIR* is a long ncRNA transcribed from the *HOXC* locus that recruits the Polycomb group (PcG) chromatin-remodeling complex PRC2 to the *HOXD* locus, where it creates a repressive chromatin environment across 40 kb [45]. However, our FISH data do not support the idea that the long ncRNAs interact with the chromatin of chromosome ends. This contrasts with the finding of Epp and colleagues, who reported that ncRNAs derived from *var* gene introns associate with the adjacent chromatin of *var* genes [18]. At this stage, we cannot exclude the possibility that a minor fraction of ncRNAs directly associate with chromatin and participate in the epigenetic control of virulence genes in *Plasmodium*. RNA immunoprecipitation, may help to determine the roles of these telomeric and subtelomeric ncRNAs in gene regulation. Furthermore, given that long ncRNAs are associated with cellular differentiation and development in many species [39,40], it is tempting to speculate that these RNAs play important roles in the transition from the schizont stage to the ring stage. Long ncRNAs also fulfill a range of other functions in cell and developmental biology, including mediating interactions with promoter elements and transcription factors to modulate transcriptional activity. At this point, we are unable to experimentally determine whether the telomeric and TARE long non-coding RNAs from *P. falciparum* are able to control gene expression in the schizont or ring stage by interacting with stage-specific promoter genes. In the future, ChIP assays may help to link lncRNAs to particular chromatin regions.

In conclusion, our work opens new avenues for studying the biological role of ncRNAs in *P. falciparum*. We demonstrated that RNA polymerase II transcribes non-coding telomeric and subtelomeric regions during the schizont stage to give rise to a family of distinct long ncRNAs in *P. falciparum*. These ncRNAs are concentrated at the nuclear periphery in a single novel compartment. Although the biological function of this compartment remains unclear, our RNA shift assays and northwestern data suggest that the TARE-6 ncRNA may recruit specific nuclear proteins to form a specialized subcompartment. Consequently, the characterization of the molecular interactions of these ncRNAs with proteins may reveal novel modes of gene regulation and nuclear function in *P. falciparum*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Long non-coding telomeric and subtelomeric RNAs are transcribed as single strands with positive polarity by a DNA-dependent RNA polymerase II. (A) Schematic representation of the telomeric and subtelomeric regions of *P. falciparum*. In this parasite, the chromosome ends are composed of the telomere plus telomere-associated repetitive elements (TAREs 1–6). The lines indicate the probes used in this work. (B) Run-on. RNA was radiolabeled, extracted and hybridized to dot blots of ssM13 DNA (3 µg) containing both the positive and negative strands of the telomeric, TARE-3 and TARE-6 regions respect to *var* gene PFI1830c. The control DNA included the gene for 28S rRNA and the vector pUC18. (C) Nuclear run-on experiments were performed with asynchronous FCR3 parasites that were incubated in the absence or presence of 0.1 mg/ml α -amanitin. Nascent [α -³²P] dUTP-labeled RNA was obtained from the nuclei of *P. falciparum* and hybridized with double-stranded DNA fragments (3 µg each) corresponding to the telomere, TARE-3, TARE-6, the DBL-1 domain of the *var1CSA* gene (RNA polymerase II control), 28S rRNA (RNA polymerase I control) and pUC18 (negative control).

Fig. 2.

Telomeric and subtelomeric regions are transcribed as long non-coding RNAs and are expressed in a stage-specific manner. (A) RNA from different asexual developmental stages of FCR3 parasites was dotted on a nylon membrane and hybridized to radiolabeled telomeric, TARE-3 and TARE-6 DNA probes. Seryl tRNA synthetase, which is expressed constitutively throughout all stages, was used as a loading control. R, rings; T, trophozoites; S, schizonts. (B) RNA from ring-, trophozoite- and schizont-stage *P. falciparum* parasites was treated with DNase I, fractionated in a 1% denaturing agarose gel and transferred to a

Hybond N+ membrane. Each membrane was hybridized to a radiolabeled telomeric, TARE-3 or TARE-6 probe. As a positive control for RNA integrity, 28S rRNA was used. Bottom, nuclear RNA was stained with ethidium bromide (EtBr) after agarose gel electrophoresis to control for loading and RNA integrity. The top schematic is a representation of the chromosome ends of *P. falciparum*. The arrows indicate the putative transcription start sites of the TARE-6 and TARE3-Telomere lncRNAs. Positive and negative strands of lncRNA studied in this work were established respect to subtelomeric *var* gene PFI1830c located on chromosome 9.

Fig. 3.

The non-coding telomeric and subtelomeric RNAs colocalize at the nuclear periphery. (A) Telomere, TARE-3 and TARE-6 transcripts were detected by using RNA-FISH assays in parasites infected in the early-schizont (30 h) and late-schizont (36 h) stage. (Top) Early-schizont stage. First row: antisense TARE-6 ss ncRNA was labeled with biotin-16-UTP (red), and antisense Telomere ss ncRNA was labeled with Alexa Fluor 488-5-UTP (green). Second row: Anti-sense TARE-6 ss ncRNA was labeled with biotin-16-UTP (red), and antisense TARE-3 ncRNA was labeled with Alexa Fluor 488-5-UTP (green). Third row:

antisense Telomere ss ncRNA was labeled with biotin-6-UTP (red), and antisense TARE-3 ss ncRNA was labeled with Alexa Fluor 488-5-UTP (green). DAPI staining is shown in blue. (Bottom) Late-schizont stage. First row: Antisense TARE-6 ss ncRNA was labeled with biotin-16-UTP (red), and antisense Telomere ss ncRNA was labeled with Alexa Fluor 488-5-UTP (green). Second row: antisense Telomere ss ncRNA was labeled with biotin-16-UTP (red), and antisense TARE-3 ss ncRNA was labeled with Alexa Fluor 488-5-UTP (green). DAPI staining is shown in blue. In each case, the merged images (yellow) show co-localization of the three small ncRNAs at the nuclear periphery. (B) ncRNA telomeric, TARE-3 and TARE-6 transcripts were detected using RNA-FISH assays in parasites infected in the ring stage. In all the case, the merged images (yellow) show co-localization of the three small ncRNAs at the nuclear periphery and only one signal was observed. At least 70 nuclei were examined per experiment two times by duplicate.

Fig. 4.

The ncRNAs define a novel subdomain in the *P. falciparum* nucleus. (A) RNA-FISH signals are shown in red, and DNA-FISH signals are shown in green (TARE-6). The signals of the TARE-6 ncRNA and telomeric DNA do not co-localize in ring stages, but in the late-schizont stage some telomere clusters are transcribed and co-localize with TARE-6 ncRNA probe. (B) *P. falciparum* nucleus present a complex nuclear architecture with several subcompartments localized in the nuclear periphery as are: telomeric clusters, *var* gene expression site, nucleolus and non-coding RNA compartment. (C) RNA-FISH signals are

shown in red (TARE-6), and 28S rDNA DNA-FISH signals are in green. The signals from 28S rDNA and the non-coding TARE-6 transcript do not co-localize. (D) Two-color RNA-FISH of a *var2CSA* RNA probe (green) and TARE-6 ss ncRNA transcripts (red) for ring stage parasites (FCR3-CSA parasite population). The signals from the *var2CSA* transcripts and the TARE-6 ncRNAs do not co-localize. In all images, nuclear DNA was stained with DAPI (blue). Scale bars: 1 μm for the ring and schizont stages. 70 nuclei were analyzed from two independent experiments.

Fig. 5.

Predicted secondary structure of the TARE-6 non-coding RNA. (A) Schematic drawing of the secondary structure predicted for 1000 bases of the TARE-6 ncRNA. (B) Predicted secondary structure of the 70 nt of TARE-6 transcript. (C) The primary sequence of the TARE-6 ncRNA used in this work is composed of 70 bases and contains three Rep20 repeats. The free energy of each stem-loop structure at 0 °C and in 3 M NaCl was calculated using RNAfold software.

Fig. 6.

TARE-6 lncRNA binds to *P. falciparum* histone H3. (A) 32 P-labeled TARE-6 RNA. probe was incubated with *P. falciparum* nuclear extract and three well-defined complexes, denoted RNA-protein complexes C1, C2 and C3 were observed by RNA EMSA assays. The specificity of these complexes was confirmed using a 100-fold molar excess of either homologous (TARE-6) or heterologous (scramble) competitors. (B) Nuclear and cytoplasmic extracts from *P. falciparum*, as well as total extracts from *E. coli* and GST protein, were subjected to SDS-PAGE, transferred to a nitrocellulose membrane, renatured

in situ and incubated with ^{32}P -labeled TARE-6 RNA probe. (C) Histones preparations from *P. falciparum* and calf and GST protein were incubated with radiolabeled TARE-6 ncRNA probe. (D) *P. falciparum* nuclear proteins were incubated with radiolabeled ncRNA transcript and anti-histone H3 antibody. The RNA–protein–antibody complexes were then analyzed by electrophoresis on a 6% native polyacrylamide gel followed by autoradiography. As a control, the same amount of anti-GST antibody did not alter the formation of these three complexes.