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Plasmodium falciparum spliceosomal RNAs: 3′ and 5′ end processing

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ABSTRACT

The major spliceosomal small nuclear ribonucleoproteins (snRNPs) consist of snRNA (U1, U2, U4 or U5) and several proteins which can be unique or common to each snRNP particle. The common proteins are known as Sm proteins; they are crucial for RNP assembly and nuclear import of spliceosomal RNPs. This paper reports detecting the interaction between *Plasmodium falciparum* snRNAs and Sm proteins, and the usual 5′ trimethylated caps on the snRNAs, by immunoprecipitation with specific antibodies. Furthermore, an unusual poly(A) tail was detected on these non-coding RNAs.

1. Introduction

The genome of *Plasmodium falciparum*, the protozoan parasite responsible for the most severe form of malaria in humans, contains nearly 5300 genes, 50% of which have introns (Gardner et al., 2002). The molecular machinery involved in splicing is thus of vital importance for the parasite's development and multiplication. Nonetheless, limited research has been done on the spliceosome.

The spliceosome is a macromolecular assembly which catalyses the removal of the intervening sequences from pre-mRNAs. It consists of five large RNA–protein complexes, called the U1, U2, U4, U5 and U6 small nuclear ribonucleoproteins (snRNPs) and many protein factors. Spliceosome snRNPs are composed of a uridine-rich RNA molecule, a core of seven Sm proteins (B/B, D1, D2, D3, E, F and G) and a set of specific proteins for each snRNA (Seraphin, 1995). The biogenesis of these snRNPs is complex and occurs in the cytoplasm. After the nuclear export of nascent snRNAs, they are recognised by the Sm proteins through a consensus sequence (PuAU4–6GPu), the Sm-site (Hermann et al., 1995). Once the heteroheptameric ring has formed around its RNA target, the 5′ cap of the snRNA is hypermethylated from a 7-methyl guanosine to a 2,2,7-trimethyl guanosine and the 3′ end is processed by endonucleolytic cleavage (Baillat et al., 2005). This leaves the mature snRNPs ready for translocation to the nucleus where the snRNAs will undergo pseudourydilation and 2′-O-methylation; the specific proteins will associate with the core snRNP (Kiss, 2004).

The 3′ poly(A) tails found on mRNA in eukaryotic cells stabilise, facilitate localisation and enhance translation; however, they could promote RNA degradation via the exosome on ncRNAs such as spliceosomal RNAs (Dreyfus and Regnier, 2002; Kadaba et al., 2004). The exosome is a complex of 3′–5′ exonucleases involved in RNA maturation and quality control (Mitchell et al., 1997). It has been shown that aberrant ncRNAs are recognised and polyadenylated by a specific poly(A) polymerase complex called TRAMP (Trf4p/Air2p/Mtr4p) which stimulates their degradation (LaCava et al., 2005; Vanacova et al., 2005). This fact suggests that 3′ end processing of ncRNAs serves as an activating factor for RNA degradation as it occurs in prokaryotic cells.

Using new bioinformatics tools for identifying ncRNAs in *Plasmodium* is fairly recent (Upadhyay et al., 2005; Chakrabarti et al., 2007; Mourier et al., 2008). Upadhyay et al., identified 18 new ncRNAs towards the end of 2005 by scanning short intergenic regions (70 pb) having more than 35% GC content and by assessing their preservation among species. Two of these 18 new ncRNAs showed features characteristic of snoRNAs and five showed sequence homology with U1, U2, U4, U5 and U6 snRNAs (Upadhyay et al., 2005). Chakrabarti et al. (2007) confirmed that *P. falciparum* predicted snRNA sequences are capable of folding into the same overall conformations as those for others snRNAs. However, experimental confirmation of bioinformatic findings was restricted to northern-blot analysis in these cases.

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Table 1
The sequence of the primers used for detecting snRNAs in *Plasmodium falciparum*. The primers were designed with "PRIMER 5" software, according to the sequence found for each snRNA.

SnRNA	Sequence		Size (pb)
pfU1	pfU1-S: ACTTACCTGGCTGGCGTTTGGG	pfU1-AS: GGCATAATCATTAGATGAATTATGC	160
pfU2	pfU2-S: CCCTTCTCGGCCTCTTGGCTAAG	pfU2-AS: GGTGCACTGTACAAACCTTTAGTGC	192
pfU4	pfU4-S: CTTTGCGGGAGGGGCAGTATCG	pfU4-AS: AGAAAGGCTACAAAAATTGCTCT	126
pfU5	pfU5-S: ACTTACTACATAACGAATCAATC	pfU5-AS: TAGGCGCGACAAAAATTCGTGGT	97
pfU6	pfU6-S: GCTCTCTTCGGAGATGCCGTTTG	pfU6-AS: ATTACATTCCTTCTCGAACGTCA	102

The current study describes *P. falciparum* spliceosomal snRNAs. Like UsnRNAs in other eukaryotes, *Plasmodium* snRNAs carry a hypermethylated cap structure on the 5′ end and they interact with Sm proteins; unusual poly(A) tails were detected on the 3′ ends.

2. Materials and methods

2.1. Culture and red blood cell lysis

P. falciparum strain FCB-2 was cultured in human O (+) erythrocytes at 37 °C in a 5% O_2, 5% CO_2 and 90% N_2 atmosphere. RPMI 1640 medium was supplemented with 25 mM Hepes, 30 mg/L hypoxanthine, 32 mM $NaHCO_3$, 50 mg/L gentamicin and 10% inactivated human serum (Trager and Jensen, 1976). Infected erythrocytes were washed twice in HBS (0.01 M HEPES, pH 7.4, 0.15 M NaCl); the parasites were released from the erythrocytes by treatment with 0.15% saponine in 10 volumes of HBS for 10 min at 4 °C. Free parasites were recovered by centrifugation at 10,000 × *g* for 10 min. The parasite pellet was washed twice in HBS.

2.2. Preparing P. falciparum cell extract

Trophozoites (2×10^9) were suspended in 1 mL TMG buffer (10 mM Tris–HCl pH 7.5, 1.0 mM $MgCl_2$, 5 mM β-mercaptoethanol and 10% glycerol) and lysed through incubation for 30 min with 0.2% NP-40 at 4 °C. Cell debris was removed by centrifugation at 14,000 × *g* for 30 min. The extract was aliquoted and stored at −80 °C.

2.3. RNA isolation and RT-PCR

Total RNA was extracted with Trizol (Invitrogen) following the manufacturer's instructions. RNA samples were DNase treated for 30 min and recovered by phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation. DNA absence in each RNA preparation was verified by PCR before carrying out RT-PCR experiments.

Reverse transcription was done in 23 μL reaction mixture (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT) with 20 ng total RNA, 0.5 mM reverse primer, 0.5 mM dNTPs and one unit of M-MLV enzyme (Promega) for 1 h at 42 °C; 0.5 mM of forward primer and 2.5 units of Taq polymerase were added to the PCR. The temperature profile consisted of: denaturing at 95 °C for 5 min followed by 30 cycles of denaturing (20 s at 94 °C), annealing (30 s, 55 °C), extension (30 s, 72 °C) and a final extension step at 72 °C for 7 min.

Polyadenylated snRNA species were detected by RT-PCR as described before; however, in this case the RNA was reverse transcribed with oligo(dT) as primer and followed by PCR with gene-specific primers (Table 1).

2.4. RNase H digestion of poly(A) tails

1 μg of total RNA was dissolved in 15 μL of 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and denatured at 70 °C for 5 min together with 100 pmol oligo$(dT)_{25}$. The reaction mixtures were incubated at 42 °C for 30 min; 2 units of RNase H (Invitrogen) were then added to each reaction and the mixtures were subsequently incubated at 37 °C for 30 min. After phenol–chloroform extraction and ethanol precipitation, the RNA was quantified and reverse transcribed as described above.

2.5. Immunoprecipitation and RNA analysis

Immunoprecipitation was performed as described previously (Steitz, 1989). Briefly, 250 μL of total extract precleared with protein-A sepharose was incubated for 2 h at 4 °C with 5 μL of Y12monoclonal anti-Sm antibody (NeoMarkers) or with 5 μL of preimmune serum (mock control). 50 μg of total RNA was incubated with 5 μL of anti-N^2,N^2,7-trimethylguanosine (αTMG) monoclonal antibody K121 (Calbiochem) for TMG-specific immunoprecipitations. After incubation, 50 μL of a 50% suspension of protein-A sepharose was added and the interaction was allowed to continue for another 2 h at 4 °C. The protein-A beads were then recovered through centrifugation and washed four times with 1 mL of TNE buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.05% NP-40). RNA was then extracted from the immunoprecipitated pellet through phenol–chloroform extraction and ethanol precipitation. The RNA was suspended in 10 μL of RNAse-free water. *P. falciparum* snRNAs present in the above suspension were amplified by RT-PCR using specific primers, as described before.

3. Results

This study was aimed at verifying whether the snRNAs reported as PfU1, U2, U4, U5 and U6 become part of the ribonucleoprotein complex. Immunoprecipitation with anti-Sm antibodies previously used for detecting *Plasmodium* Sm proteins (Francoeur et al., 1985) and anti-trimethylated cap antibodies was thus carried out. snRNAs reported as PfU1, U2, U4, U5 and U6 were immunoprecipitated with anti-Sm and anti-TMG antibodies in our experimental conditions, whereas messenger RNA, Pf TERT, known to lack the Sm association and the hypermethylated cap structure, was not detected in the same immunoprecipitates (Fig. 1). All RNA samples were DNase treated and DNA absence was verified by PCR. These results in conjunction with a prior study (Bawankar et al., 2010) indicated that a trimethylguanosine cap structure was present at the 5′ end of the snRNAs and that they formed stable ribonucleoparticles *in vivo*. U6 snRNP contains a set of Lsm proteins (Sm-like) and its RNA molecule is monomethylated (Singh and Reddy, 1989); since it forms a functional hybrid with U4, it could be pulled down indirectly by antibodies directed against the other snRNPs (Achsel et al., 1999).

Immunoprecipitates were tested with primers to amplify a different non-coding RNA, PfTER (Telomerase RNA). TER is capped with a trimethylguanosine cap on its 5′ end in *Saccharomyces cerevisiae* and is associated with Sm proteins; consequently telomerase RNP in budding yeast is similar to the spliceosomal RNPs (Seto et al., 1999); none of these features had been tested before in *P. falciparum*. PfTER was not immunoprecipitated by the Sm antibody but it was recognised by the anti-TMG antibody (Fig. 1). The hyperme-