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Transcript maturation in apicomplexan parasites

Elena S. Suvorova^{1,*} and Michael W. White²

Elena S. Suvorova: essuvorova@gmail.com; Michael W. White: mwhite.usf@gmail.com

¹Center for Drug Discovery and Innovation, University South Florida, Tampa, FL 33612

²Department of Global Health, University South Florida, Tampa, FL 33612

Summary

The complex life cycles of apicomplexan parasites are associated with dynamic changes of protein repertoire. In *Toxoplasma gondii*, global analysis of gene expression demonstrates that dynamic changes in mRNA levels unfold in a serial cascade during asexual replication and up to 50% of encoded genes are unequally expressed in development. Recent studies indicate transcription as well as mRNA processing have important roles in fulfilling the “just-in-time” delivery of proteins to parasite growth and development. The prominence of post-transcriptional mechanisms in the Apicomplexa was demonstrated by mechanistic studies of the critical RNA-binding proteins and regulatory kinases. However, it is still early in our understanding of how transcription and post-transcriptional mechanisms are balanced to produce adequate numbers of specialized forms that is required to complete the parasite life cycle.

Keywords

Toxoplasma gondii; *Plasmodium* spp; gene expression; mRNA splicing

Introduction

Unicellular eukaryotic parasites of the Apicomplexa phylum have complex lifestyles that involve propagation in different hosts accompanied by major morphological transformations needed to move between hosts. Parasite survival greatly depends on changes of gene expression and in *Toxoplasma* ~2–5% of encoded genes are thought to be uniquely expressed in each developmental stage [1, 2]. A remarkable serial order of gene expression is also associated with parasite replication with nearly 40% of mRNAs cyclically expressed that is thought to deliver proteins in a “just-in-time” sequence to daughter parasites [3, 4]. The profiles of cyclical mRNAs are particularly dramatic in the S and mitotic periods

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*Address correspondence to: Elena S. Suvorova, Florida Center for Drug Discovery and Innovation, University South Florida, 3720 Spectrum Blvd, Suite 304, Tampa, FL 33612, essuvorova@gmail.com, Office (813) 974 5796, Fax (813) 974 0992.

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(including cytokinesis) of the parasite cell cycle when many specialized structures and invasion organelles are produced *de novo*.

Our understanding of how gene expression in the Apicomplexa is regulated has significantly improved in recent years. In parasite development there is a clear evidence of classical mechanisms based on cis-trans regulation of a core promoter complex, which involves the activity of large families of plant-related AP2 transcription factors [4–8] and chromatin remodelers [9, 10]. By contrast, the mechanisms responsible for cell cycle mRNA profiles in the asexual stages is less understood and here the role of ApiAP2 factors is much less clear. Nearly 75% of ApiAP2 factors with peak expression in the S/M periods of the *Toxoplasma* tachyzoite cell division are dispensable (White and Hong, unpublished results) and some late cell cycle ApiAP2 factors have exclusive roles in development [8]. A partial answer to this paradox may lie downstream of transcriptional initiation. There are extensive RNA-based machineries in these parasites responsible for transcript maturation (splicing, mRNA capping and polyadenylation) and mRNA stabilization (protection, degradation) that may act in accord with translational control (RNP granules, μ ORFs, and mi-RNAs) in order to coordinate cell cycle gene expression profiles. There are excellent reviews of Apicomplexa translation control [11], therefore, here we will focus on nuclear mRNA processing with emphasis on the splicing machineries and their regulation.

Whole cell mRNA analysis

Recent deep RNA sequencing has confirmed the extensive cell cycle mRNA cascade that unfolds in these parasites [12–15] and has also provided a more complete view of the RNA landscape that has aided gene annotation, identified alternative splice sites, and precisely mapped 3' and 5' UTR ends [15–17]. New groups of RNA were identified by these efforts including long non-coding, anti-sense RNA [12, 15, 18, 19] and a wealth of the small RNAs [15, 20]. These last discoveries are not yet understood given that parts of the RISC machinery are absent from Apicomplexa genomes [20, 21].

Overall RNA sequencing has discovered ~20% more transcripts [12, 15, 16] with ~5% of genes in *Plasmodium falciparum* producing an average 2–4 or more transcripts [15]. Different types of alternatively spliced (AS) mRNAs were detected with alternative donor and acceptor types being predominant and transcript truncation the largest fraction of AS-transcripts in *P. falciparum* [12, 15]. These findings explain the presence of nonsense-mediated mRNA decay (NMD) machinery in Apicomplexa genomes [15, 18], which is widely used by other eukaryotes to regulate protein abundance. Importantly, these results indicate that the Apicomplexa produce futile RNAs that are likely regulated by NMD process. Whole-genome assessment of AS-transcripts has also revealed unexpected linkage between different levels of gene regulation. For example, AS-forms of *P. falciparum* PF3D7_0103200 and PF3D7_0601200 mRNAs associate exclusively with loaded ribosomes [18], denoting selective use of the alternative transcripts at the certain stage of the parasite life. To understand how widespread these mechanisms are we will need to characterize the translated transcriptome in each parasite.

Major splicing machinery in Apicomplexa

Assembled genomes of Apicomplexa species reveal different gene organization with introns present in 5% of genes in *Cryptosporidium* as compared to >75% of genes in *Toxoplasma* (Fig. 1A). Intron sequences in *P. falciparum* [15, 22] and *Toxoplasma* [23–25] have the canonical 5' GU-AG 3' splice junction, and because of the AT-rich genome, *P. falciparum* has uniquely replaced the canonical G in the 5th position of the 5' splice site with no apparent reduction in splicing efficiency [22, 26]. Apicomplexa introns have significant nucleotide variations at the branch point and there is an indication of additional regulatory elements located in introns and exons [22]. RNA-binding proteins of heterogeneous ribonucleoprotein (hnRNP) and serine/arginine-rich (SR) family are widely used in other eukaryotes to modulate the splice site recognition via binding to enhancer or silencer sequences [27, 28] and many of these factors are encoded in Apicomplexa genomes (Table S1) [29]. In model eukaryotes these splicing regulatory elements are known to contribute significantly to the splicing specificity, although their role in apicomplexan parasites has not yet been evaluated.

Removal of intron sequences from pre-mRNA is executed by the multicomponent complex called the spliceosome [30], which is comprised of five major small nuclear RNP complexes; U1, U2, U4, U5 and U6 snRNPs. At the core of each complex is uridine-rich snRNA bound to Sm-like proteins and a number of RNP-specific RNA-binding factors. In *P. falciparum*, spliceosomal RNAs are abundantly expressed, modified by methylation and polyadenylation, and folded into canonical structures critical to assemble into functional snRNP complexes [26, 31, 32]. Major protein components of the spliceosome were identified by *in silico* analysis in several apicomplexan species [15, 29, 32, 33] (Fig. 1B, Table S1) with the level of conservation observed following the hierarchy; U5 > U4/U6 > U2 > U1 (Table S1; e-values shown in parenthesis). Several splicing factors have low similarity implicating specialized evolution of splicing in the Apicomplexa, and in *Toxoplasma*, the spliceosome complement includes a second U2AF65 paralog that is exclusively expressed in the sporozoite stage (not shown).

Regulation of the conserved mRNA splicing

The studies of Apicomplexa splicing are few, and therefore, little is known about the functions of divergent splicing factors or how these mechanisms might regulate gene expression. Our characterization of the cell cycle mutants in *Toxoplasma* has identified a novel RNA-binding protein, TgRRM1, that is required for G1 phase progression in the tachyzoite [29]. Instability of the mutant TgRRM1^{Y169N} protein at high temperature led to immediate arrest in the early G1 phase and caused mis-splicing of nearly all intron containing genes [29]. The complete disruption of RNA processing inputs was unexpected as this is not observed in other eukaryotes where the competitive nature of constitutive splicing [30] and the abundance of splicing factors (80–100th percentile RMA in Apicomplexa species, eupathDB) leads to late or random growth arrest [34–37]. The loss of TgRRM1 from the U4/U6.U5 tri-snRNP complex may explain the disruption of splicing (Fig. 2A) [29], however, we do not understand yet how this leads to the rapid arrest of tachyzoites in G1 (<1 hr, Fig. 2B and C) as transcription is active throughout the parasite

cell cycle [4]. TgRRM1 may directly influence the G1 cyclin/CDK master regulators, which in higher eukaryotes is the provenance of pocket proteins and the conserved transcription factor E2F [38]. We might speculate that without these known G1 regulators, RNA-level regulation may have replaced these functions in apicomplexan parasites. Consistent with this idea, our study of a large collection of *Toxoplasma* temperature-sensitive mutants has identified many RNA-binding proteins that are essential cell cycle regulators (Suvorova and White, unpublished results).

Regulation of the alternative mRNA splicing

Apicomplexa parasites have complex life styles often involving multiple hosts and the corresponding large protein complement reflects the diverse morphological stages required. To expand protein repertoire eukaryotes have adopted complex exon-intron gene structures that provides for expanded gene expression through alternative splicing. Abundant AS transcripts in *Plasmodium spp.* create functional isoforms of surface proteins (for review see [39]), stage-specific proteins [40] and proteins involved in intra erythrocytic development cycle [14, 18]. In *Toxoplasma*, mRNA products have been detected for genes that play essential roles in host cell invasion, metabolism, cytoskeleton, regulation of cell division and development [12, 41–46].

In eukaryotes, RNA-binding proteins of the hnRNP and SR family are actively involved in regulating AS production [27, 28]. Direct binding to the regulatory sequences in exons and introns (see above) determines availability of the splice site by either enhancing or inhibiting spliceosome assembly. Apicomplexa parasites also encode this capacity with an exceptional expansion of the RRM-domain family (1% of total genes) that includes conserved SR and hnRNP proteins [29]. Despite the abundance of RNA-binding proteins and potential importance to parasite biology there has been limited research on these factors (TgDRE1 [23, 47]; PfSR1 [48, 49]). Alternative splicing is regulated by phosphorylation of the SR factors by cdc2-like kinases (CLK) of the CMGC family. Apicomplexa genomes encode four members of CLK kinase family [48, 50]. Given the homolog of PfCLK-1/Lammer and PfCLK-2 kinases to yeast splicing kinase Sky1p it was expected these kinases would have a role in alternate splicing. The *Plasmodium* ortholog of Sky1p substrate Npl3p protein, splicing factor PfASF-1, was actively phosphorylated by these essential kinases [50], however, the link between regulation of the alternative splicing is yet to be established. Another member of the *Plasmodium* CLK family of kinases, PfSRPK1 not only phosphorylated splicing factor PfSF1 but showed inhibitory effect on RNA splicing in nuclear extracts [48].

Conclusions

We are at an early stage in our understanding the mechanisms controlling gene expression in apicomplexan parasites. What is well established is the role of steady-state mRNA levels in providing the scale and timing of protein expression. There are cases where the direction and magnitude of these changes do not match, however, this is more exception than rule [18, 51–53]. What we don't fully understand are the mechanisms changing mRNA levels during parasite growth and development. Here we have much work to do. The focus over the last

decade has been on promoter regulation and in developmental gene expression the principles are emerging from the studies of ApiAP2 factors [4–7] and chromatin remodelers [10]. This may explain up to 50% of gene expression leaving us with sizable mysteries about how constitutive mRNAs are maintained and how the dynamic cyclical transcripts of the replication stages are rapidly produced. There is evidence that post-transcriptional RNA mechanisms have important influences on parasite gene expression. Extensive anti-sense transcription and evidence for microRNAs have been documented in these parasites, although the biological ramifications of these RNA inputs remain elusive [12, 15, 20]. More important are the mechanisms affecting mRNA metabolism where nuclear and/or cytoplasmic processes can rapidly influence mRNA levels as has been demonstrated in the cell cycle transcriptome of *P. falciparum* merozoites (plasmoDB) [54]. RNA processing, degradation and utilization (translation) are the least understood influences on protein expression in these parasites and future studies of gene expression will need more focus on the mechanisms downstream of transcriptional initiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Gene expression mechanisms downstream of transcriptional initiation in Apicomplexa is highlighted.

Deep RNA sequencing has revealed a wealth of RNA processing in Apicomplexa.

Parasites have complete splicing machinery, but, how splicing is regulated remains to be determined.

Global control of mRNA splicing in *Toxoplasma* unexpectedly linked to G1 phase checkpoints.

Figure 1. Spliceosome machinery in Apicomplexa phylum

A. Percentage of the genes with introns was determined in four model apicomplexans (*Toxoplasma gondii*, *Plasmodium falciparum*, *Thieleria parva*, *Cryptosporidium parvum*) and compared to the relatively low-intron content of yeast (*Saccharomyces cerevisiae*).

B. Spliceosome components present in Apicomplexa genomes; major U1, U2, U4/U6 and U5 snRNP complexes were analyzed. Orthologs of human splicing factors were identified by pBLAST and gene IDs and e-values are listed in Supplemental Information (Table S1). Existing orthologs are indicated with an X, absent components are indicated with an O.

Figure 2. Novel splicing factor TgRRM1 associates with the U4/U6.U5 tri-snRNP complex and controls early steps of G1 phase

A. Association of the *Toxoplasma* factor, TgRRM1 with tri-snRNP complex of spliceosome may explain the global mis-regulation of splicing in ts-TgRRM1 mutant parasites. The precise role for TgRRM1 in spliceosome assembly and/or function is not understood. Two possibilities in assembly function are indicated with red arrows.

B. *Toxoplasma* tachyzoites carrying a defect in TgRRM1 rapidly arrest with a 1N genomic content and no mitotic structures at high temperature (not shown), which when combined with the elevation of early G1 transcripts is consistent with a specific cell cycle block in the first half of G1 [29]. G1 transcripts were categorized based on the peak expression in cell cycle transcriptome [4] following G1a - early G1 (0–2h in the cycle); G1b - late G1 (2–4h in cell cycle). Percentage of the up- and down-regulated transcripts from each G1 category is shown.

C. The mRNA profiles of 3 representative up-regulated (G1a red) and down-regulated G1 (G1b green) transcripts illustrate the dramatic changes in cell cycle gene expression in TgRRM1 depleted cells.