A genomic glance at the components of the mRNA export machinery in *Plasmodium falciparum*

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**Key words:** helicase, malaria parasite, nucleus, nuclear pore, RNA transport

Nuclear export of mRNAs is one of the steps critically important for gene expression and different steps of mRNA processing are linked to the export of the mRNA out of the nucleus. This coupling probably provides a quality control mechanism as well as a higher efficiency for the synthesis of mRNAs. The mRNA is synthesized in the nucleus and then exported to the cytoplasm through the nuclear pore complexes (NPCs), which are embedded in the nuclear envelope. The Mex67- Mtr2 complex in yeast and its counterpart Tap-p15 in higher eukaryotes function as an mRNA exporter through the NPC. Some of the DEAD box proteins such as UAP56 and Dbp5 have been implicated in mRNA export also. In this report using the bioinformatics approach we have analyzed the components of the mRNA export machinery in *Plasmodium falciparum* and also highlighted the salient features of some of the components. Further detailed studies on various components of nuclear mRNA export in *Plasmodium falciparum* will be essential to understand this important pathway.

The nucleus is a membrane-bound compartment and the defining feature of all eukaryotes. It encloses the genome and provides a distinct spatial environment for two of the most fundamental processes of replication and transcription, separating them from the protein translation in the cytoplasm. The access to the nuclear compartment during interphase is restricted due to the presence of a double lipid bilayer called the nuclear envelope (NE). In spite of this physical segregation, there is a constant shuttling of macromolecules between the nucleus and the cytoplasm, so much so that in a growing cell 1 million macromolecules may be transferred between the two compartments every minute.1,2 This directional nucleocytoplasmic exchange is highly specific and regulated, enabling the export of ribosomal RNAs, micro RNA (miRNA), transfer RNA (tRNA), small nuclear RNA (snRNA) and messenger RNA (mRNA) transcripts into the cytoplasm and the import of many proteins required for genome stability, replication, transcription, splicing, export and a host of other processes into the nucleus. This directional transport is a critical cellular process, which influences the gene expression and signal transduction events.

Nuclear export of mRNAs is one of the steps critically important for gene expression. The mRNA is synthesized in the nucleus and then exported to the cytoplasm through the nuclear pore complexes (NPCs), which are large proteinaceous channels embedded in the NE. The overall shape and architecture of the NPC is highly conserved from yeast to higher eukaryotes and these are the only gateway for the transport and exchange of macromolecules between the nucleus and cytoplasm. It is interesting to note that the machinery for export is highly conserved from yeast to humans. NPCs are dynamic octagonally symmetric macromolecular complexes, which mediate nucleocytoplasmic communication and allow shuttling of proteins and ribonucleoprotein complexes (RNPs) and selective transport of cargoes through their central aqueous channel.3,4 NPC are large complexes of the size ranging from 60 Mda in yeast to 125 Mda in vertebrates and harbour multiple copies of 30 to 50 different proteins called Nucleoporins or Nups.5-7 Nups are essential and critical molecular components required for the assembly and function of the NPC. The number of Nups per cell is highly variable and depends on the cell size and activity. Yeast cells have ~200 NPCs/nucleus and a mature Xenopus oocyte has about 5 x 10^7 NPCs/nucleus.8 A typical mammalian cell may have as many as 2,000–5,000 NPCs depending on the cell type and growth conditions. To facilitate the import and export of large molecules nuclear transport receptors are required but ions, metabolites and other small molecules of less than 40 kDa can passively diffuse through the aqueous channel of the nuclear pores. Larger macromolecules require soluble nuclear receptors to bind and facilitate the transport of cargo through the NPCs.9,10 Based on the presence or absence of hydrophobic phenylalanine-glycine (FG) repeats, Nups can be categorized into ‘unstructured’ or ‘structured’ forms. The physical barrier imposed by the NPC is due to the presence of a meshwork of FG Nups in the lumen of the channel pores,10,11 FG Nups are natively unfolded subset of Nups, which line the channel pores of the NPC and form a sieve like barrier by interlinking their hydrophobic FG repeat domains.10,12 For mRNA export the binding of export factors to FG motifs (FXFG, GLFG and FG) of NUPs is necessary.13 Nups that are devoid of FG repeats are structured and constitute the backbone
Facilitated nuclear transport is an energy dependent and signal mediated process requiring transport factors belonging to a family of conserved proteins, collectively known as karyopherins (Kaps). These proteins are nuclear transport receptors belonging to the importin β family of HEAT repeat proteins and they are further dependent on the biological activity of a cofactor, the GTPase Ran. Soluble nuclear transport receptors (NTRs) continuously shuttle between the two compartments through the NPCs either alone or together with the cargo macromolecules. The transport receptors bind cargo destined for translocation either directly or via an adapter, recognizing certain targeting signals such as Nuclear Localization Signals (NLSs) and Nuclear Export Signals (NESs) to facilitate the transport of cargo through the NPC. Moreover, the sequence and the substructural content of NLSs and NESs determine their specificity for various Kaps. The passage of the cargo bound receptor complexes through the NPC requires specific interactions with the FG Nups. The FG sequence repeats of Nups serve as a potential docking site for Kaps and these Kaps further provide a biochemical selection mechanism for nucleocytoplasmic transport (NCT). Moreover, mutations in the transport receptors rendering them defective for binding to the hydrophobic pockets of FG Nups are known to impair their nuclear translocation. This NTR—cargo complex is then translocated through the pores of NPC and cargo is released in the destined compartment in an energy dependent manner. Multiple independent import and export pathways have a unifying theme where NTRs perform multiple roles of cargo recognition, translocation through the nuclear pore and cargo release in the destined compartments. Two nucleotide states of the small GTPase Ran, which is predominantly present in the nucleus, play an important role in the directionality of NCT. The binding of RanGTP triggers the association of exportin: cargo complexes and the dissociation of importin: cargo complexes. In the nucleus, RanGTP functions to release the cargo from its import receptor by binding to the receptor itself. In the nucleus, exportins bind to their cargo in the presence of GTP and this complex is transported back into the cytoplasm. The GTP hydrolysis at the cytoplasmic NPC face promotes the disassembly of the export receptor-cargo complex in the cytoplasm and this causes the recycling of the export receptor and restores the cytoplasmic RanGDP pool.

Nuclear microinjection of tRNA in the Xenopus oocyte first revealed that the overall process of RNA export across the nuclear envelope is a carrier mediated translocation process, that is saturable and temperature sensitive. Subsequently it became apparent that different RNA species (mRNA, tRNA, miRNA and rRNA) are exported out of the nucleus in a mutually exclusive manner, where one RNA species doesn’t saturate the export of another in a coinjection experiment. It is well established now that the export of tRNA, miRNA, (sn)RNA and rRNA follows this general scheme that involves exportins of the kaps family and the Ran cycle. But the general mRNA export is mechanistically different as it uses a transport receptor that is unrelated to Kaps and is independent of the Ran cycle. Furthermore numerous additional export factors such as adaptors and release factors cooperate with the mRNA export receptor. The export of tRNA is carried out by the export receptor importin-β family member Exportin-t, which binds tRNA in a RanGTP-dependent manner. miRNA and tRNA are exported by the karyopherin exportin-5 and Ran/Crm1 export pathway respectively. The nuclear export of RNA is mediated by proteins but unlike the transport of proteins, members of the Kap superfamily are not involved in mRNA export. mRNA transcripts are transported from the nucleus to the polysomes in the cytoplasm in the form of large ribonucleoprotein (RNP) complexes. There are constant changes in mRNP composition as these mRNPs progress from synthesis to processing and finally to export.

Components of the mRNA Export Machinery in P. falciparum

SR proteins and the TREX complex. The SR (Ser/Arg-rich) proteins are one such family of splicing factors, which can serve as adaptors proteins by recruiting the export factor TAP/NXF to mRNA destined for export. These SR proteins contain one or two RNA recognition motifs (RRMs) and Ser/Arg/-rich domain that can be phosphorylated at multiple positions. Ubiquitously found protein complexes like the TREX (transcription/export complex) are involved in coupling activities like the transcript elongation, the splicing and the export. TREX complex is a multbsubunit complex consisting of THO (suppressor of the transcriptional defect of Hpr1 by overexpression) subcomplex, Tex1 and two additional export factors UAP56 (Sub2 in yeast
and UAP56 in metazoa) and Yra1p or ALY/REF adaptor (Yra1 in yeast and ALY or REF in metazoa).42,43 The components of the TREX complex such as Hpr1p are able to recruit the export receptor to the mRNAs by recruiting the export factors Aly and UAP56.43 UAP56 is loaded by components of the TREX complex and it in turn recruits the export receptor TAP/Mex67 by the interaction with ALY/REF.40,44

TREX complex is a highly conserved protein complex involved in the export of mRNA in eukaryotes. In yeast, the TREX complex is made up of a THO subcomplex consisting of Tho2, Hpr1, Mft1, Thp2, and the export factors Sub2 and Yra1 along with Tex1, a protein of unknown function.42 The TREX complex is itself loaded cotranscriptionally to the elongating transcript and is further responsible for recruiting many proteins involved in the process of splicing and mRNA export. SR proteins like Gbp2, Hrb1 and Npl3, are recruited to mRNA by the TREX complex.42 The components of the TREX (THO) complex are responsible for recruiting Grb2 and Hrb1 but not Npl3, which is recruited independently by the interaction with components of the cap binding complex. The shuttling SR proteins apart from carrying out splicing and export by acting as export adapters for the mRNA export factors.40 Npl3 has been shown to provide directionality to the process of export by undergoing a cycle of phosphorylation and dephosphorylation events as described previously.

BLAST search (www.ncbi.nlm.nih.gov) using the S. cerevisiae proteins Npl3 and Gbp2 as query in the P. falciparum database “PlasmoDB” (www.plasmodb.org/) revealed two proteins with PlasmoDB number PF10_0217 and PF10_0068 respectively. These proteins PF10_0217 and PF10_0068 are annotated in the PlasmoDB as pre-mRNA splicing factor and RNA binding protein respectively. Moreover, using bioinformatics approaches we were not able to detect the homologue of the SR protein Hrp1 in P. falciparum. PF10_0217 and PF10_0068 contain 538 and 246 amino acids and show considerable similarity to the yeast proteins Npl3 and Gbp2 respectively. The expression data reported in PlasmoDB show that both of these proteins are expressed in all the developmental stages of the parasite. PfNpl3 (PF10_0217) homologue contains two RRMs in the N terminal region of the protein (Fig. 1A) similar to its human homologue (Fig. 1B). PfGbp2 (PF10_0068) homologue surprisingly has only two RRMs (Fig. 1C), whereas the corresponding human homologue, heterogeneous nuclear ribonucleoprotein has three RRMs (Fig. 1D) and is quite larger in size than its counterpart in apicomplexans. The difference in the homologous proteins of P. falciparum and other higher eukaryotes is due to the divergence during the course of evolution. The structural modelling of the RRMs of the PfGbp2 was done using the RNA binding protein Fir as the template.45 The results of this modelling show that there is significant structure conservation in the RRMs of the two proteins as the two structures are completely superimposable (Fig. 1E).

In P. falciparum, although some of the components of the TREX complex like Tho2, UAP56 (PfU52) and REF are present, but using the bioinformatics approach we were unable to detect the rest of the components of the TREX complex. It has been reported previously that all the components of the TREX
Moreover, PfTho2 is unannotated and has been described as a conserved hypothetical protein of unknown function in PlasmoDB (www.plasmodb.org). The expression data reported in PlasmoDB show that this protein is expressed in all the developmental stages of the parasite. The corresponding homologues in P. vivax (PVX_101385) and P. yoelii (PY01809) are also unannotated hypothetical proteins.

UAP56 is a bona fide splicing factor involved in the export of mRNA transcripts. It directly interacts with the N and C terminal of ALY to recruit it to the spliced mRNPs. Apart from Tho2, UAP56 homologue (PfU52) and Ref/Aly (described separately) are also present in the P. falciparum’s TREX complex.

UAP56 is a member of DEAD box family of RNA helicase, involved in the ATP dependent assembly of spliceosome. UAP56 is an essential protein, that has been implicated in the export of mRNA and it has been shown recently that P. falciparum homologue is an RNA dependent ATPase and it also has a role in the splicing processes. In yeast it was observed that there is a rapid accumulation of poly(A) RNA upon shifting of the temperature sensitive Sub2/UAP56 mutants to non permissive temperatures.

In Drosophila also double-stranded RNA (dsRNA) mediated depletion of HEL/UAP56 leads to growth inhibition and robust accumulation of poly(A) RNAs in the nucleus. The structural modeling of the PfU52 (Fig. 2A) was done using the human UAP56 (Fig. 2B) as the template and it was observed that although the overall structure is conserved but insertions in the protein tend to loop out (Fig. 2C).

REF (RNA and Export Factor Binding Proteins) Family

ALY/REF (Yra1p in yeast) is an evolutionary conserved family of hnRNP-like proteins, called REF (RNA and export factor binding proteins), which plays the role of an adaptor protein between REF and mRNPs. Yra1p was originally identified as a yeast nuclear protein which exhibits RNA annealing activity. Multiple members of the REF family have been reported only in Mus musculus, Xenopus laevis, Caenorhabditis elegans, Schizosaccharomyces pombe and Saccharomyces cerevisiae. In Saccharomyces cerevisiae the two members of the REF family are known by the names of Yra1 and Yra2. Yra2p when overexpressed is able to complement the deletion of YRA1 in vivo, suggesting that these proteins have redundant functions.

In P. falciparum PSI-BLAST® search (www.ncbi.nlm.nih.gov) using the full length protein of yeast Yra1 as a query revealed an ORF with PlasmoDB number PFF0760w, showing ~28% identity and ~55% homology with the yeast protein. Interestingly, PFF0760w shows even more similarity to its human homologue ALY, showing ~34% identity and ~62% homology.
binding (REF) protein, whereas the corresponding homologues in *P. vivax* (PVX_113920) and *P. knowlesi* (PKH_113330) are annotated as RNA binding proteins. The expression data reported in PlasmoDB show that this protein is expressed in all the developmental stages of the parasite. Orthologues of *P. falciparum* REFp (PlasmoDB number PFF0760w) are annotated as hypothetical proteins in *P. yoelii* (PY05533) and *P. berghei* (PB102089.00.0). Although only one member of the REF family is present in the *P. falciparum* and *P. vivax* but our bioinformatics analysis reveals that two members of the REF family are present in both *P. yoelii* (PY07541 and PY05533) and *P. berghei* (PB102089.00.0 and PB406000.00.0). Surprisingly, the proteins of the REF family from *Plasmodium* species range between 100–160 amino acids and are smaller than their eukaryotic counterparts, which contain about 200–300 amino acids. Moreover, REF family proteins are characterized by the presence of a central RNA binding domain (RBD), which is flanked on both the sides by two conserved N and C terminal domains. The conserved N and C terminals of the REF proteins are separated by variable insertions of positively charged amino acids. In *Plasmodium* species, the RBD of the REF family is located more towards the C terminal end with a conserved N terminal domain. The C terminal domain of REF in apicomplexans is inconspicuous and is almost fused to the RBD (Fig. 3A and B). The structure of PfREF was modelled and the results show that the structure is almost similar to the RNA binding motif of the template 2E5H (Fig. 4). The structure of the template was downloaded from the MMDB database.

**Tap-p15 Pathway**

To transport mRNPs through the NPCs, the yeast Mex67-Mtr2 complex and the homologous metazoan TAP-p15 complex
help of its NTF2 like fold to form heterodimer complex. In P. falciparum database PF14_0305 is annotated as the homologue of the nuclear mRNA export factor TAP. This homologue (PF14_0305) shows ~21% identity and significant similarity (1.4e-05) with the yeast export factor Mex67. The corresponding homologues of TAP in P. vivax (PVX_084925) and P. berghei (PB300366.00.0) are annotated as conserved hypothetical proteins. PF14_0305 and PVX_084925 have a very long N terminal region with insertions of arginine and lysine and are about twice the size of the other eukaryotic counterparts. The modular architecture of PF14_0305 (PfTAP homologue) is not clear as the NTF2 and UBA domains are not apparent in the bioinformatics analysis using the InterProScan sequence search software. The absence of the modular architecture in PF14_0305 might be due to the sequence divergence during the course of evolution. It is important to note that the Nxt1/P15 homologue is also absent in P. falciparum and other apicomplexans as revealed by the PSI-BLAST search. Nxt1 homologues are apparently present in fungi and other higher eukaryotes but they are absent in protozoans.

Remodelling ATPase Dbp5 and its Substrates

It has been suggested that two DEAD-box helicases Dbp5 and UAP56 play key roles in the process of RNA export and are indispensable for this export. RNA helicases are ubiquitously involved in each step of RNA metabolism. Dbp5 determines the overall directionality of the process of mRNA export. The activation of Dbp5 in turn is controlled by IP6 (Inositol hexa-phosphate)—bound Gle1 at the cytoplasmic side of the NPC. This activation most likely facilitates the remodeling of mRNP protein composition during directional transport and provides energy for transport cycles. The weak ATPase activity of Dbp5 is stimulated by the cellular cofactors like Gle1 and IP6. Recombinant CTD (C terminal domain) of Gle1 activates Dbp5 by decreasing the Km of the enzyme for ATP approximately about six fold and side by side increases the Kcat about four-fold, resulting in the overall increase in the kinetic efficiency of the enzyme. The stimulation of Dbp5 is in turn required for RNA unwinding and displacement of proteins from RNA-protein complexes. Gle1 binds to Nup42 and anchors to the cytoplasmic fibrils of NPC at a site, which is juxtaposed to the Dbp5-Nup159 binding site. Dbp5 is a yeast RNA helicase involved in remodelling RNPs extruding out of the NPC by removing Mex67 and other hnRNP like proteins from the exported mRNP. In a Dbp5 mutant, there is an increased association of Mex67 with the mRNAs, suggesting a direct role of this protein in RNP remodelling at the nuclear rim. Most of the physiological substrates of Dbp5, like Mex67 and other mRNP bound proteins tend to accompany the mRNP into the cytoplasm but are not found in association with the polysomes. All of these results suggest that these accompanying proteins are removed upon entry into the cytoplasm from the NPC, just prior to translation. It has been shown that the bound proteins are displaced by the ADP bound form of Dbp5. Recently, it has been shown that Nab2p is one of the physiological substrate for Dbp5p. Moreover, Nab2p bound RNPs are targeted by Dbp5p at the cytoplasmic

Figure 4. The computer based structure modeling of (A) PfREF of Plasmodium falciparum based on the (B) 2E5 h-template (C) The superimposed image. (also known as NXF1-NXT1) function as general mRNA export receptors. Mex67 and TAP bind poly(A) RNA and show genetic and biochemical interactions with the components of the NPC, thus exhibiting characteristic features of the export receptors. In yeast, the functionally homologous Mex67 partner in RNA export is Mtr2 whereas in higher eukaryotes Tap and p15 form a heterodimer complex. Tap/Mex67 is the principal export factor involved in eukaryotes which mediates the export of the bulk of mRNA from the nucleus. In yeast there is a single Tap/NXF homologue known as Mex67 but in higher eukaryotes there are multiple members of the NXF family formed by separate gene duplication events. Tap/Mex67 is a modular protein having three distinct domains: a leucine rich repeat domain (LRR), a NTF2 like middle domain and a C terminal UBA (ubiquitin associated fold). Mex67p heterodimerizes with Mtr2p with the

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The orthologous proteins of \textit{P. vivax} (PVX\_114270), \textit{P. yoelii} (PY03499) and \textit{P. berghie} (PB000664.00.0) are also described as hypothetical proteins. Furthermore the results of the InterProScan (www.ebi.ac.uk) of the corresponding (PFF1110c, PfNab2) protein revealed the presence of two Zinc finger CCCH type motif (from amino acid 543 to 568 and 569 to 589) and a conserved panther domain from amino acid 525 to 785 (PTHR14738) (Fig. 5). The results of our analysis show that the Zinc finger CCCH type motif is only present in the orthologous proteins of \textit{P. vivax}, \textit{H. sapiens}, \textit{C. elegans} and absent in the \textit{S. cerevisiae} homologue Nab2. Moreover \textit{P. falciparum} and \textit{P. vivax} homologues of Nab2 also contain a PWI domain at their N terminal (Fig. 5). A PWI domain is a novel nucleic acid binding motif and the proteins bearing this motif are involved in the processes like transcription, 5’-end capping, splicing, 3’-end processing (cleavage and polyadenylation), surveillance, turnover, transport and translation.\textsuperscript{76} Since the PWI domain is absent in the human as well as the yeast orthologues, we propose here that PfNab2 might be playing roles other than the ones played by Nab2 protein of yeast and it might be a key protein involved in the RNA metabolism.

### Concluding Remarks

It is well established that the export of mRNA from the nucleus to the cytoplasm takes place through a well defined pathway. Export
competent mRNP assembly is coupled to transcription and this mRNP is composed of mRNA and a number of other RNA export factors. These export factors include Mex67-Mtr2, Npl3, poly(A) binding protein (PABP) and the DEAD-box protein Dbp5. Dbp5 is responsible for the release of mRNA from mRNP in the cytoplasm. The knowledge of mRNA export mechanisms in malaria parasites is very limited and in its infancy. We have already initiated the characterization of some of these components such as PABP\(^{7,8}\) and Dbp5 (Mehta and Tuteja, manuscript in preparation) from *P. falciparum*. The comparative studies reported here have laid the foundation for future work and further detailed studies on the various RNA export machineries in the malaria parasite are essential to better understand this important process.

**Acknowledgements**

The work in RT’s laboratory is supported by grants from Department of Biotechnology, Defence Research and Development Organization and Department of Science and Technology. Infrastructure support from the Department of Biotechnology, Government of India is gratefully acknowledged.

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