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Is mitochondrial gene expression coordinated or stochastic?

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Abstract

Mitochondrial biogenesis is intimately dependent on the coordinated expression of the nuclear and mitochondrial genomes that is necessary for the assembly and function of the respiratory complexes to produce most of the energy required by cells. Although highly compacted in animals, the mitochondrial genome and its expression are essential for survival, development and optimal energy production. The machinery that regulates gene expression within mitochondria is localized within the same compartment and, like in their ancestors, the bacteria, this machinery does not use membrane-based compartmentalization to order the gene expression pathway. Therefore, the lifecycle of mitochondrial RNAs from transcription through processing, maturation, translation to turnover is mediated by a gamut of RNA-binding proteins, all contained within the mitochondrial matrix milieu. Recent discoveries indicate that multiple processes regulating RNA metabolism occur at once but since mitochondria have a new complement of RNA-binding proteins, many evolved *de novo* from nuclear genes, we are left wondering how coordinated are these processes? Here we review recently identified examples of the coordinated and stochastic processes that govern the mitochondrial transcriptome. These new discoveries reveal the complexity of mitochondrial gene expression and the need for its in-depth exploration to understand how these organelles can respond to the energy demands of the cell.

Introduction

The ancestral prokaryotic origin of mitochondria has contributed to some of the unique features of mitochondria as we know them today. Many genes of the ancestral mitochondrion have been integrated into the nuclear genome and as a consequence the mitochondrial genome has been significantly compacted, particularly in animals where the mitochondrial DNA (mtDNA) is minimal and its genes are free of introns. Nevertheless, mtDNAs have retained their circular organization akin to bacterial genomes in most organisms. Typically, mitochondrial genomes produce their own set of tRNAs, rRNAs, and mRNAs that encode a subset of the protein subunits of the electron transport chain (ETC) complexes [1]. In animals, the heavy and light strands of the mtDNA are transcribed as large, polycistronic transcripts where the mRNAs and rRNAs are typically separated by tRNAs that act as punctuation marks [2]. These mitochondrial transcripts are regulated at the post-transcriptional level by diverse families of RNA-binding proteins (RBPs) such as the pentatricopeptide repeat (PPR) and Fas-activated serine/threonine kinase (FASTK) families [3–5] and other unique RBPs. The regulation of mitochondrial gene expression by RBPs has been reviewed extensively previously [6–8]; and here we highlight recent discoveries that question if its regulation is coordinated, where a series of proteins work synergistically, or stochastic, which is less controlled and subject to random events and biological noise.

A transcript is born

Near-genome size transcripts are produced by the mitochondria specific RNA polymerase (POLRMT) that requires the presence of transcription factor A (TFAM), transcription factor 2B (TF2BM), and the transcription elongation factor (TEFM) [9,10]. The structure of POLRMT resembles T7 RNA polymerase but has an N-terminal extension and two pentatricopeptide repeats (PPRs) [11]. Recent studies have shown that this N-terminal extension functions to suppress transcription at non-promoter sites and that this suppression can only be relieved by TFAM [12]. Structural insights into the initiation of transcription show that TFAM binds to the promoter region and recruits POLRMT, relieves its inhibited state, and enables the recruitment of TF2BM [12]. TF2BM induces a conformational change in POLMRT to move an intercalating hairpin structure to separate DNA strands and this interaction is stabilised through the C-terminal region of POLMRT [9]. Once initiation is complete, TFAM and TF2BM dissociate, allowing TEFM to interact with the intercalating hairpin structure to facilitate transcription elongation of near-genome length transcripts [9,13,14]. TEFM stops the formation of an RNA G-

quadruplex structure that forms upon the transcription of a G-rich sequence near the heavy strand origin of replication (oriH), termed the conserved sequence block (CSB) [14] Newly synthesised RNA exits towards the PPR-containing domain via the RNA exit channel in the N-terminal region [14]. The transcribed RNA in this G-rich region forms a hybrid structure large enough to obstruct the RNA exit channel of POLRMT, resulting in the premature termination of RNA synthesis [14]. While this action prevents gene expression, it is suggested that the short RNA sequences that are produced in the absence of TEFM binding can act as the primers for mtDNA replication [15]. Recent studies using *Polrmt* knockout mice showed reduced mtDNA levels and *de novo* mtDNA synthesis rates, confirming the role of POLRMT in the synthesis of RNA primers *in vivo* [16]. Additionally, it was shown that POLRMT preferentially initiates transcription at the light strand promoter *in vivo* under low POLRMT levels, likely facilitating a switch mechanism between RNA primer formation, maintenance of mitochondrial DNA copy number and gene expression [16]. Because production of both the polycistronic mitochondrial transcripts and the short RNA primers that are required for DNA replication are mutually exclusive, at least at the molecular level, the modulation of this process has been described as a regulatory switch between transcription and DNA replication. The presence of TEFM biases this equilibrium towards full-length transcription, however how this is regulated *in vivo* is not currently understood. For example, once POLRMT has stimulated the initiation of DNA replication, via RNA primer production, could subsequent transcription complexes perform efficient transcription of genome length RNAs using the same mtDNA molecule, or vice versa? The recent structural studies of the mitochondrial transcription initiation complex reveal that the complex is distinct from the nuclear, bacterial and bacteriophage counterparts. Mitochondria have evolved to rely on a defined set of unique proteins that are all non-redundant and essential and it remains to be determined how this well-orchestrated process of transcription initiation and elongation is regulated under different energy demands.

Coordinated biogenesis of RNAs?

In animal mitochondria the polycistronic transcripts produced by POLRMT require extensive processing, stabilization and maturation to produce individual RNAs. In recent years, evidence has emerged that numerous proteins involved in RNA processing, modification, and mitochondrial ribosome (mitoribosome) assembly are localising in discrete foci that have been termed mitochondrial RNA granules (MRGs) [17–19]. MRGs have been shown to associate with mtDNA-nucleoids, leading to the hypothesis that RNA may co-transcriptionally associate with MRG proteins to begin endoribonucleolytic processing [17]. Recently our group identified that

mitochondrial RNA processing *in vivo* is hierarchical, where the 5′ end cleavage of tRNAs by the RNase P complex precedes 3′ tRNA cleavage by ELAC2, the mitochondrial RNase Z enzyme [20–22]. The order of processing was determined by knocking out the endonuclease component of the RNase P complex, MRPP3 (also known as PRORP), that associates with the two additional proteins, MRPP1 and MRPP2 (also known as TRMT10C and HSD17B10, respectively), and most recently by knocking out ELAC2 [21,22]. Detailed transcriptomic analyses identified that impaired 5′-end tRNA cleavage compromises subsequent 3′-end tRNA cleavage in the *Mrpp3* knockout mice; and in the *Elac2* knockout mice, loss of 3′-end tRNA cleavage did not impair 5′-end tRNA cleavage. This suggests that the unique mitochondria-specific RNase P complex has evolved to resolve the complex polycistronic transcripts in animals, whereas the evolutionarily conserved ELAC2 enzyme can only recognize its substrates once the complex RNA structures have been resolved and smaller RNA substrates released by the RNase P complex. The ordered processing indicates that this aspect of mitochondrial gene expression is coordinated between the two enzymes.

Structural analysis of MRPP3 showed that the active site is in a conformation where key metal binding residues are bound by other amino acids; rendering it catalytically inactive [23,24]. This is a key difference between MRPP3 and the *Arabidopsis thaliana* homolog PRORP1 where these amino acid interactions are not present [23,24]. This has led to the hypothesis that MRPP3's interactions with the MRPP1/2 complex, and possibly a pre-mt-tRNA substrate, alter the conformation of its active site allowing for endonuclease activity [24]. MRPP1/2 have been shown to remain bound to RNA that had been processed by MRPP3, and this continued binding improves the efficiency of ELAC2 cleavage of the majority of mitochondrial tRNAs (mt-tRNAs) *in vitro* [25]. This supports *in vivo* findings that correct 5′ tRNA processing is required prior to 3′ tRNA processing by ELAC2. MRPP1/2 was also retained on mt-tRNA after the 3′ CCA addition, suggesting that MRPP1/2 can act to stabilise mt-tRNA interactions with modification enzymes to facilitate their maturation [25] and the cooperation between the three subunits of the RNase P complex is necessary for its function in animal mitochondria.

Another surprisingly coordinated feature of mitochondrial gene expression was identified using proteomic analyses of mitoribosomes fractionated into small and large subunits as well as assembled monosomes that revealed the co-transcriptional assembly of the large mitoribosomal subunit [21]. In both *Mrpp3* and *Elac2* knockout mice unprocessed 16S rRNA transcripts were bound by early assembling mitoribosomal proteins of the large subunit suggesting coordination between mitochondrial transcription and early assembly of the mitochondrial ribosomes

[21,22,26,27]. However, it is interesting to note that an analogous partially assembled RNA-protein complex is not observed for the unprocessed 12S rRNA, demonstrating that the pathways for small and large mitoribosome subunit assembly are mechanistically distinct.

Another component of the MRGs, FASTKD5 was recently identified to regulate RNA processing of the non-canonical regions that are not flanked by tRNA genes in the mtDNA [28,29]. Knockdown of *Fastkd5* caused impaired processing of the non-canonical junctions *mt-Nd5-cyt b* and *mt-Atp8/6-Co3* [29], and future work will determine if this regulation is exerted by facilitating processing or stabilising the mature products and if FASTKD5 can act as a platform to recruit yet to be discovered enzymes that carry out the cleavage of these transcripts. Nevertheless, defects in processing upon *Fastkd5* knockdown resulted in impaired protein synthesis and mitoribosome assembly [29], further indicating that RNA processing is co-regulated with mitoribosome assembly and protein synthesis.

To stabilize and protect

Proteins that stabilize and modify mitochondrial RNAs are also found within the MRGs. Recently, we identified that the pentatricopeptide repeat domain protein 1 (PTCD1) is essential for survival because it is required for *mt-16S* rRNA stability and maturation via its association with FASTKD2 and the RNA pseudouridylate synthase domain containing 4 protein (RPUSD4) [30]. *Ptcd1* knockout mice had reduced 16S rRNA pseudouridinylation, loss of the large ribosomal subunit, impaired mitoribosome assembly, and loss of protein synthesis [30]. Recent studies have localized PTCD1 and RPUSD4 to the MRGs along with FASTKD2 [18], and knockout cell lines for *Rpusd4* and *Fastkd2* have reduced levels of the 16S rRNA, albeit not as dramatic as those found in the *Ptcd1* knockout mice [18,30–32]. These observations indicate that biogenesis of the large ribosomal subunit is regulated by a complex composed of PTCD1, RPUSD4, and FASTKD2 that controls 16S rRNA modification, where loss of any constituent proteins can destabilise the complex and decrease protein synthesis. The requirement for scaffold of RNA-biding proteins that recruit modification enzymes on the 16S rRNA suggests a new mode of stabilization that is unique to mitochondria but the evolutionary and mechanistic requirements are not yet understood.

The leucine-rich pentatricopeptide repeat cassette protein (LRPPRC) interacts with the SRA stem-loop interacting RNA-binding protein (SLIRP) and is essential for maintaining mitochondrial mRNA polyadenylation, stability and coordinated protein synthesis [33–35]. Our recent footprinting and PAR-CLIP analyses of LRPPRC-SLIRP binding sites revealed that this

complex is a mitochondrial RNA chaperone required for relieving secondary structures in mRNAs to facilitate protein synthesis [36]. LRPPRC binding sites were also identified within the bicistronic mRNAs *mt-Nd4l/4 and mt-Atp8/6* exemplifying the need for this protein complex to expose internal start and stop codons of bicistronic mRNAs and facilitate translation by the mitoribosome [36]. The RNA chaperone role of LRPPRC explains its close association with the mitochondrial poly(A) polymerase (MTPAP), which is necessary for polyadenylation of the 10 mitochondrial mRNAs encoded by the heavy strand of the mtDNA, and the PNPase/SUV3 degradasome, which exhibits the 3′ exonuclease activity [37]. The stochastic action of MTPAP has been observed in RNA-Seq data where poly(A) tails have been identified on the mitochondrial rRNAs, tRNAs and after addition of the CCA nucleotides on the 3′ end of mature mt-tRNAs [38]. This spurious activity of the MTPAP can be mitigated by the exonuclease PDE12 that was recently shown to specifically remove incorrectly added poly(A) nucleotides from mt-tRNAs and rRNAs [39]. Circularisation methods to capture and deep sequence long precursor transcripts that accumulate when mt-tRNA cleavage is impaired enabled us to identify that processing occurs concurrently with degradation and polyadenylation of RNAs [20]. This unexpected finding provides clues that these particular processes are stochastic in mitochondria and directed by biochemical kinetics as opposed to functioning in concert with other regulatory enzymes. Although stochastic processes exist within mitochondria, clearly the introduction of new functions and enzymes, such as this deadenylating maturation pathway, helps to recover the productive maturation of mitochondrial RNAs.

While LRPPRC acts to stabilise mitochondrial mRNAs through polyadenylation, the FASTK protein has been shown to specifically stabilise the only non-polyadenylated mRNA, *MT-ND6* [40]. FASTK can act in concert with the degradasome complex to direct the maturation of the *MT-ND6* mRNA by binding its 3′-UTR to protect it from the degradasome's exonuclease activity, while the degradasome removes the antisense-ND5 RNA from the 3′ end of the transcript [40]. Studies of the FASTK protein family [4], much like the PPR protein family [41], have revealed diverse roles in mitochondrial RNA regulation. *FASTKD1* knockout cells have a specific increase in *MT-ND3* mRNA levels and *FASTKD3* knockout cells have increased levels of heavy strand encoded mt-RNAs [28,42]. Both FASTKD1 and FASTKD3 have additional roles in regulating translation rates, indicating a possibly dual mechanism of action for regulating gene expression. Recent structural modelling of the RAP domain of FASTK proteins predicted that they may also adopt an endonuclease structure, which could possibly

account for the increased levels of certain mRNAs in knockout cells and provide an alternate model for the function of FASTK family proteins [28]. Clearly the introduction of mitochondria-specific RBPs has helped shape the regulation of the mitochondrial transcriptome and it now remains to be determined how the functions of these complexes are coordinated under varying energy pressures.

A "plug and play" protein synthesis mechanism

Currently it is not clear how mitoribosomes recognise mRNAs for translation since mitochondrial mRNAs lack 5′ leader sequences [43]. Cryo-electron microscopy has revolutionized our understanding of the mitoribosome and has revealed potential mechanisms for its recognition of mRNAs. In response to the massive evolutionary pressures imposed on mitochondrial translation by the massive compaction of mtDNA, and rRNAs in particular, mitoribosomes have acquired a large number of additional, or supernumerary, protein components. One of these supernumerary ribosomal proteins, the PPR protein mS39 (also known as MRPS39 or PTCD3 [44]), is positioned near the mRNA entrance site on the ribosomal small subunit and recent investigation has shown that it associates with *mt-Co3* mRNA [45]. It appears that mS39 may facilitate the association of mRNA with the initiation complex by recognition of mRNAs and their delivery to the decoding site of the mitoribosome.

Recent studies into the translational activator of cytochrome c oxidase subunit 1 (TACO1) have unearthed how *mt-Co1* mRNA is recognised by the mitoribosome. TACO1 was shown to associate with the small ribosomal subunit of the mature mitoribosome, specifically regulating the translation of COXI and, as a consequence, complex IV assembly and activity [46]. TACO1 was found to adopt a hook conformation with a negatively charged patch in its N-terminal domain binding to the *mt-Co1* mRNA[46].

The mitochondrial ribosomal protein of the small subunit 27 (mS27 or MRPS27) is another PPR-containing supernumerary mitoribosomal protein. MRPS27 was identified at the base of the small subunit and was shown to participate in forming bridges between the small and large subunits highlighting its essential role in mitochondrial translation from previous studies [47–49]. The hydrophobic nature of mitochondrial proteins has led to the model that the mitoribosome may interact with the mitochondrial inner membrane allowing for co-translational membrane insertion of synthesized proteins. The mitochondrial ribosomal protein of the large subunit 45 (mL45 or MRPL45) has been suggested to mediate an interaction between the mitoribosome and the inner mitochondrial membrane via its C-terminal domain

[50]. Surprisingly an extension of the N-terminal region of mL45 was shown to occupy the polypeptide exit tunnel, indicating that this extension must be displaced for proteins to exit the ribosome. This extension was shown to be essential for translation and hypothesised to mediate interactions between the mitoribosome and the membrane bound protein insertase OXA1, allowing co-translational insertion of newly synthesised mitochondrial proteins into the inner membrane [45]. The mitochondria-specific ribosomal proteins reveal new evolutionary adaptations that coordinate the peculiar demands of mitochondrial protein synthesis.

Conclusions and future work

Recent advances in our understanding of the role of RNA-binding proteins in mitochondrial transcription, RNA processing, RNA maturation, and translation illustrate the many different genetic regulatory modes and their interdependent natures. POLRMT works by complexing with TFAM, TF2BM, and TEFM in a step-wise manner to recognise the DNA promoters, unwind DNA, and synthesise near-genome size transcripts. MtDNA has been shown to be in proximity to the recently characterised MRGs, which are centres of RNA processing and maturation. How the complex of MRPP1/2 acts as a platform to facilitate RNA endonuclease processing by MRPP3 followed by ELAC2 illustrates a well-regulated hierarchy for RNA processing events. The synergistic actions of RNA maturation enzymes further fine tune expression by regulating processes like RNA stability through polyadenylaion and ribosome assembly by rRNA maturation. The regulation of ribosomal recognition of mRNA and timed membrane insertion of nascent polypeptides facilitate efficient production of the respiratory complexes. However, as well as these coordinated events, the observations of simultaneous RNA processing and degradation, as well as gratuitous polyadenylation of non-target mitochondrial RNAs, demonstrate that mitochondrial gene regulation also incorporates stochastic processes that rely on biochemical kinetics to create balanced gene expression. Therefore, mitochondrial gene expression appears to balance both forms of gene regulation. Further work will unravel the remaining components regulating coordinated mitochondrial gene expression as well as mechanisms to tolerate or repair the consequences of inefficient stochastic events and how they all interact to enable efficient energy production.

Abbreviations

ELAC2, ElaC domain protein 2; ncRNAs, non-coding RNAs; OXPHOS, oxidative phosphorylation; RNA-Seq, RNA sequencing; snoRNAs, small nucleolar RNAs.

Author contributions

All authors contributed to writing and editing the manuscript. D.R. created the figure.

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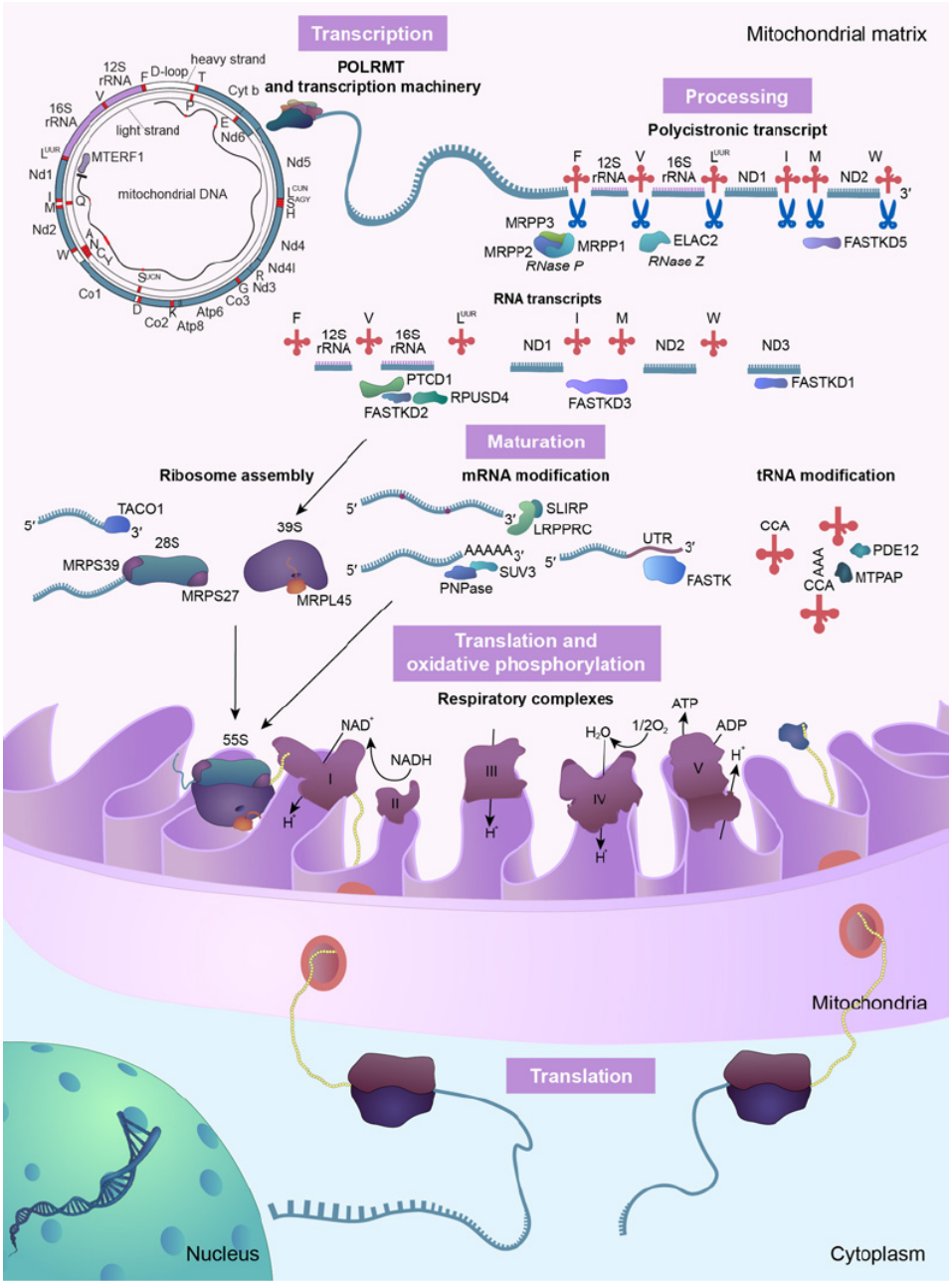
Conflict of Interest

The authors declare no conflict of interest.

Figure 1



The RNA-binding proteins that regulate mitochondrial gene expression. While not compartmentalised, many components involved in mitochondrial gene expression co-localise to create hotspots of regulatory activity. The localisation of these gene expression components facilitates both the coordinated pathways, which utilise a series of interdependent protein complexes, and the stochastic processes, where several proteins are required to overcome the somewhat random nature of these proteins' actions.

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