

Terminating human mitochondrial protein synthesis

A shift in our thinking

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Until recently, human mitochondria were regarded as unusual as they appeared to employ four stop codons to terminate translation. In addition to the UAA/UAG of the universal genetic code, two arginine triplets (AGA/AGG) had been re-assigned as termination signals. This posed the conundrum of what factor was responsible for recognizing these triplets to promote translation termination? Recent data indicates that in fact no protein is required to recognize AGA/AGG. Indeed, it is the absence of any cognate factor, tRNA or polypeptide that is important. On encountering either of these 'hungry' codons at the end of an open reading frame, instead of requiring a novel or modified release factor, human mitoribosomes employ -1 frameshifting to reposition a standard UAG codon in the A-site, indicating that only the universal UAA and UAG are used as stop codons. This renders a single mitochondrial release factor, mtRF1a, previously shown to be capable of terminating 11 of the 13 open reading frames encoded by the mitochondrial genome, to be sufficient to release all nascent human mitochondrial gene products from the mitoribosome.

Protein synthesis is a fundamental cellular process, decoding template messenger RNA (mRNA) in the ribosome to produce polypeptides. Sequence elements within the mRNA dictate where the ribosomal subunits are to be recruited, where translation should commence and where it should stop. Many of these cis-acting elements, together with the trans-acting factors that cooperate with the ribosomes controlling

this procedure, have been identified and characterized, revealing the existence of numerous differences between archaea, eubacteria, the eukaryotic cytosol and certain organelles within. One such organelle is the mitochondrion, which contains its own genome and the required apparatus for intraorganellar protein synthesis. Interestingly there appear to be differences even between mitochondria from different organisms. In this report, we focus on protein synthesis in human mitochondria, specifically on the apparent recoding of stop codons and translation termination.

After initiation, elongation continues to drive the mRNA through the ribosomes until a stop codon is translocated into the A-site. These codons are aptly named as once these are positioned in the A-site the ribosome has to 'stop', as generically there are no tRNAs that will recognise these triplets, thus preventing further elongation of the polypeptide. Sequence specificity is still required but triplet recognition is effected by proteins rather than tRNA. These proteins are termed release factors (RF), of which there are two types, class I and II. Stop codon specificity is exhibited by the class I RFs, whilst class II stimulate their release factor activity. Class I RFs contain three domains that facilitate codon specificity or promote the release of the newly synthesized protein from the ribosome. Stop codon recognition is achieved through interactions between the A-site triplet and two motifs of the RF.¹ The termination triplets in the universal genetic code are UAA, UAG and UGA. The eukaryotic cytosol and archaea each employ a single species of RF, often described as omnipotent, which

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Abbreviations: mt-mRNA, mitochondrial messenger RNA; mt-DNA, mitochondrial DNA; hmt-ribosomes, human mitochondrial ribosomes; ORF, open reading frame; RF, release factor; bp, base pairs

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Table 1. Comparative alignment of the conserved GGQ motif of eubacterial RF1/RF2 with the human mitochondrial release factor family

Protein	Sequence spanning GGQ motif	UniProt ref. no.
<i>E. coli</i> RF1	PADLRIDTF-RSSGAGGQHVNTT-DSAIRITHLPT	P0A710
<i>E. coli</i> RF2	PADLRIDVY-RTSGAGGQHVNRT-ESAVRITHIPT	P07012
Human mtRF1	PKDLRIDTF-RAKGAGGQHVNKT-DSAVRLVHIPT	O75570
Human mtRF1a	PKDLRIDTK-RASGAGGQHVNTT-DSAVRIVHLPT	Q9UGC7
Human c12orf65	DENELEEQFVKGHG PGGO ATNKT-SNCVVLKHIPS	Q9H3J6
Human ICT1	PLDRLTISYCRSSGPGGQ NVNKNVNSKA EV R FHLAT	Q14197

can recognise all three stop codons.²⁻⁴ Eubacteria, in contrast, use two proteins; RF1, recognizing UAA and UAG, and RF2 with specificity for UAA and UGA. All of these RFs use two domains to elicit this selectivity, a tripeptide domain (designated PXT in RF1 types and SPF in RF2 types) and the region at the tip of the α -5 helix.¹⁵ These two domains come together in space in close proximity to the stop codon to effect recognition. Peptide release itself requires the third domain, with the strict requirement for a GGQ motif.⁶ Following codon recognition, this motif is brought close to the peptidyl transferase centre within the ribosomal large subunit, promoting hydrolysis of the ester bond linking the P site terminal tRNA with the nascent polypeptide leading to peptide release.^{5,7}

To every rule there is an exception and mitochondria, in particular human mitochondria, behave just a little differently. These organelles contain their own genome (mtDNA) encoding the two mt-rRNAs required for human mitoribosome (hmt-ribosome) biogenesis, thirteen open reading frames (ORFs), and all twenty two mt-tRNAs that are sufficient to facilitate intra-organelle translation of these mt-ORFs.⁸ Following the sequencing of the human mtDNA several perceived recodings were predicted: AUA to methionine, AUU can also be recognized by initiator $tRNA^{Met}$, and UGA specifying tryptophan.⁸ One other striking alteration was the apparent recoding of AGA/AGG from arginine to termination codons. Why were AGA/AGG predicted to be reassigned as stop signals? Not one of the twenty two mtDNA encoded mt-tRNAs contained an anti-codon that could decode either of these triplets. Further, human mitochondria do not normally import tRNAs from the cytosol.⁹ As a result human mitochondria were thought to employ four

stop codons; UAA, UAG, AGA and AGG leading to the question of what the release factor requirement would be. Certainly, as no standard release factor had been identified that would recognise AGA/AGG, one would predict the existence of a new form of RF. Would this new protein be an omnipotent RF such as eRF1, or would human mitochondria follow the eubacterial paradigm of two release factors with different specificities?

Twelve years ago the issue was apparently resolved when mtRF1 was identified.¹⁰ Alignment with a number of RF1 type factors from a variety of organisms indicated an amino acid sequence identity of between 30 and 40%, making mtRF1 a strong candidate.¹⁰ As would be required of an RF, the GGQ motif is present in mtRF1 and the mini-domain in which it is positioned appears to be conserved (Table 1). Both of the domains involved in sequence recognition, however, are somewhat divergent in sequence and length but this was assumed to allow specificity for this expanded and unusual repertoire of four termination signals in human mitochondria.^{10,11} Subsequent biochemical and in vivo analyses, however, failed to reveal any release activity associated with this protein.^{12,13} After further bioinformatic mining, a second candidate emerged. This protein had greater similarity in sequence and length to each of the two sequence recognition domains present in RF1- rather than RF2-type proteins and was therefore termed mtRF1a. Both in vitro and in vivo analyses confirmed that mtRF1a was active on UAA and UAG triplets but no activity could be detected with AGA/AGG codons positioned in the 70S *E. coli* ribosomal A-site.¹¹ This accounted for termination of eleven of the thirteen human mt-ORFs. It did not, however, resolve the question of which protein was responsible for release of polypeptides encoded by

MTCO1 and *MTND6*, the two remaining mt-ORFs that terminate in AGA and AGG, respectively. Recent experiments from our laboratory have finally uncovered the molecular mechanism by which termination of these two transcripts is effected.¹² As described below, the mt-ORFs terminating in AGA or AGG also use mtRF1a to promote release of the nascent polypeptide but not through direct recognition of the AGA/AGG triplets.

On inspection of sequences surrounding the putative AGA/AGG stop codons it was apparent that both are directly preceded by a 'U'. By invoking a -1 frameshift, hmt-ribosomes could position UAG in the A-site, thereby requiring only the standard stop codons of UAG and UAA for peptide release (Fig. 1). Frameshifting, however, is not a common phenomenon in mitochondria. A limited number of examples have been identified from sequence analysis but these examples explain how protein elongation is maintained on a broken open reading frame by a shift in the +1 direction.¹⁴⁻¹⁶

To determine whether hmt-ribosomes do indeed use a -1 frameshift to terminate these mt-ORFs, it was necessary to map termination codons in exquisite detail. For this, we employed RelE, an endonuclease that cleaves codons between nucleotide positions 2 and 3 in the *E. coli* ribosomal A-site.¹⁷ Further, it also has a high affinity for UAG codons in the A-site, but a low affinity for AGA and AGG triplets. A human cell line was engineered to target RelE to mitochondria (mt-RelE). We confirmed that on mitochondrial import, mt-RelE retained not only ribosome and A-site specificity but also that cleavage occurred only between residues two and three of the A-site codon. On examination of mitochondrial protein synthesis post mt-RelE expression, we observed not only the expected decrease in the translation of

transcripts terminating in UAA or UAG but also a decrease in translation of both transcripts carrying either AGA or AGG, namely *MTCO1* and *MTND6* respectively. As RelE has a very low affinity for AGG and non detectable K_{cat}/K_m values for AGA,¹⁷ this de novo synthesis data provided indirect evidence that neither of these codons were positioned in the ribosomal A-site at termination and that -1 frameshifting is employed in both cases. To confirm directly that this was the case, subsequent fine mapping of the triplet resident in the A-site during translation termination of *MTCO1* was performed as detailed in.¹² Briefly, this was done by isolating RNA from cells post induction of mt-RelE, then trapping the 3'-termini by ligating on a DNA oligonucleotide, followed by specific reverse transcription and PCR regimes.¹² Cloning of these species revealed the precise sequences generated upon mt-RelE cleavage of the A-site substrate. This confirmed unequivocally that UAG is shifted into the A-site.¹² As a result of these data, it has become clear that the use of AGA and AGG as stop signals was an understandable misinterpretation of the original primary sequence data.⁸ The striking consequence of this frameshifting is that human mitochondria use only the two universal stop signals UAA and UAG and so conform to the majority of mitochondrial translation systems, requiring only a single mitochondrial release factor.¹⁸ Hence, the human mitochondrial release factor, mtRF1a, is sufficient to promote cleavage of the ester bond between the terminal P-site tRNA and the nascent peptides encoded by all thirteen open reading frames in human mitochondria.

Programmed ribosome frameshifting is more commonly associated with bacterial and viral genomes where many examples have been extensively characterized.¹⁹ It is a strategy that allows the ribosome to forward or back translocate in a directed manner that is not dictated by in-frame triplets, commonly these are -1 or +1 frameshifts. To slip the ribosome along a transcript requires the breaking and remaking of codon/anticodon pairings. The tension forces causing the slip can be influenced by both the transcript sequences upstream and/or downstream of the slip site (reviewed in refs. 20 and 21). Downstream

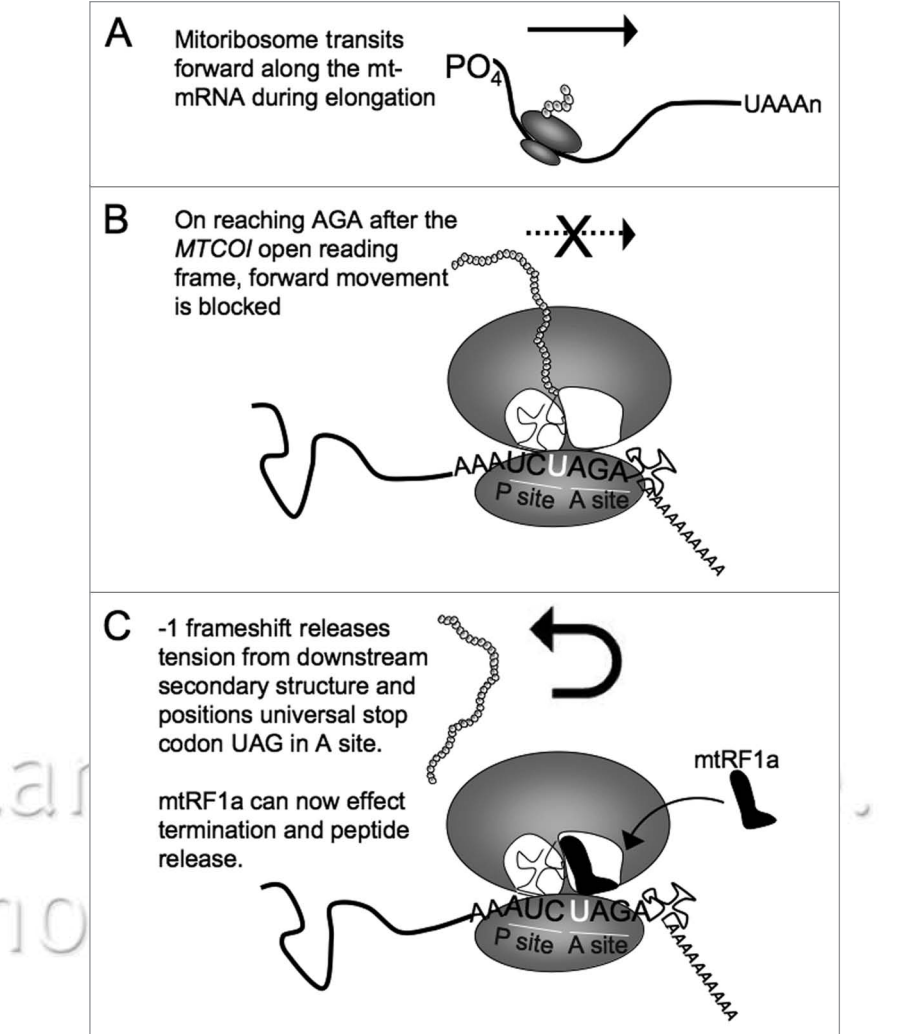


Figure 1. Human mitochondrial ribosomes frameshift in the presence of AGA and AGG codons. During normal elongation the hmt-ribosomes translate the mt-mRNA as it moves through the 55S monosome (A). Upon reaching an AGA/AGG codon, the 55S particle collides with a stable secondary structure generated, in the case of *MTCO1* by the antisense of the mt-tRNA^{SER(UCN)}, downstream of the triplet and in the absence of any mt-tRNA or protein factor that can recognise the A-site codons (B), the hmt-ribosome performs a -1 frameshift (C). This results in the positioning of a universal stop codon UAG in the A-site, which can now be recognized by mtRF1a and the nascent polypeptide is released (C).

sequences can form stable secondary structures, which in part impede the progress of the ribosome. Upstream of the shift site so called 'slippery sequences' also impact on the probability of frameshifting and, in bacteria, interactions between 16S rRNA and upstream mRNA sequence may also play a part. Physiological conditions and feedback loops can also influence whether the ribosomes continue to read through or frameshift, and readthrough is generally favoured over slippage.²² Thus, the tension forces generated will be dependent on physiological conditions, the type of

ribosome (70S, 80S or 55S), the sequences of the rRNA and mRNAs involved and will either enhance or inhibit the propensity to frameshift.

How does this relate to human mitochondrial transcripts? In each of the two mt-ORFs that contain the hungry codons AGA or AGG, the preceding nucleotide is a U. Thus, by back tracking the ribosome by a single base in the -1 direction, it is possible in each instance to position UAG codons into the A-site to restore use of a conventional stop signal. This arrangement appears to be longstanding

in hominids since it is conserved in the mtDNA sequence of Neanderthal and the recently determined Siberian Denisova hominid.^{23,24} Downstream of both *MTCOI* and *MTND6* there are secondary structures, which are generated by the 3'UTR, a feature not common in human mt-mRNAs. Although these 3'UTR sequences are predicted to form secondary structures that may be strong enough to stimulate a frameshift, analysis of the sequences immediately flanking this human mitochondrial frameshift site indicate that they do not conform to the currently defined consensus for upstream 'slippery sequences' or downstream spacers separating the paused ribosome and the downstream inhibitory structure. However, these human mitochondrial mRNAs are not alone in their divergence from the reported consensus sequence characteristics. There are an increasing number of examples where upstream sequences clearly function to promote frameshifts but do not match the convention and the same is true for the downstream spacers.^{20,25}

Irrespective of the differences in the human mt-mRNA sequences compared to the frameshift consensus, a critical feature is absence of cognate mt-tRNAs for AGA and AGG codons, making these 'hungry' or even 'starving' codons. These codons play a critical role despite not being conventional stop codons, precisely because these triplets are not recognized by either a release factor or an mt-tRNA. Their function is to remain empty and cause the hmt-ribosome to pause before slipping back by a nucleotide and thus contribute to a novel -1 frameshift mechanism.¹²

Despite the poor similarity in the flanking sequences between human *MTCOI/MTND6* and the consensus, these sequences are sufficient to drive a frameshift. Disappointingly, however, since it is currently not possible to manipulate the human mitochondrial genome, we cannot precisely delineate the cis-acting elements that contribute to the frameshift. Another factor that is likely to be critical to creating permissive conditions for the frameshift, is the mitoribosome itself. The mammalian mitoribosome has been studied in great detail, but not exclusively, by the groups of O'Brien, Spremulli and Agrawal. These

groups have documented just how significantly different this particle is, not only when compared to the bacterial 70S and the eukaryotic 80S ribosome but also in comparison to other mitochondrial ribosomes.²⁶⁻²⁹ The normal ratio of ~70% RNA to 30% protein is reversed, and this increased protein content affects the buoyant density and sedimentation value generating a 55S mitoribosome. Although the 55S and bacterial 70S particles have a similar mass, those of the mammalian organelle are larger and have a more porous structure.²⁹ Further, on isolation the mammalian mitoribosome large subunit has a strong tendency to retain P-site tRNA, which is in contrast to E-site tRNA that co-purifies with bacterial ribosomes. This together with the fact that the mammalian 16S mt-rRNA has lost regions corresponding to the rRNA of the bacterial 70S ribosome that would contact the E-site tRNA, has led to the suggestion that these mitoribosomes lack an E-site (reviewed in ref. 26). This would have an impact on the ease of 55S frameshifting, as with an AGA or AGG in the A-site there would be only a single anti-codon/codon pairing in the P-site to break and reform.

What makes this example of frameshift novel? In addition to being the first example of frameshifting in mammalian mitochondria, it is also unusual in that it does not cause a frame change in order to continue translation of the same open reading frame, as mentioned above. It is also unusual as this frame change is not induced in order to commence translation of an overlapping open reading frame. This is a common strategy utilized by viruses to maximise genetic information from a minimal genome.^{30,31} Intriguingly, human mtDNA does encode two overlapping bicistronic units. How does the downstream start codon recruit a mitoribosome in these bicistronic RNA units? This question has been very difficult to address because of the intractability of mitochondria to transfection and the lack of an in vitro translation model. Suggestions have included the use of an internal ribosome entry site (IRES), a strategy that is also common to viral gene expression. However, in light of the observation that human mitoribosomes can frameshift, it is interesting to speculate whether

frameshifting is invoked to initiate translation of the downstream open reading frame. In the bicistronic unit encoding *MTND4L/MTND4*, the mitoribosome would need to reverse by only a single nucleotide position from the MTND4L termination site to position the AUG start codon into the P-site. In the case of the second unit, *MTATP8/MTATP6*, a shift of approximately minus forty would be required. Although the latter distance between stop and start is longer, perhaps it is not beyond the realms of possibility as there are examples of ribosomal hopping that span 50 nucleotides with absolute specificity and precision (reviewed in ref. 19). This issue of whether the mitoribosomes do in fact slip or hop back to reinitiate and translate the downstream ORF is one that we are currently addressing. Finally, perhaps the most distinctive feature of the hmt-ribosome frameshift is that in contrast to all other examples that we are aware of, readthrough in the absence of a frameshift cannot occur as there is no tRNA or protein that has been identified that can recognise the AGA or AGG codons in human mitochondria.

It is now confirmed that to terminate translation of all the human mt-ORFs only mtRF1a is required, however, the mitochondrial release factor family has four assigned members, mtRF1, mtRF1a, ICT1 and c12orf65.³² Curiously, although flanking regions contain some variation, all four of these proteins retain the GGQ motif (Table 1), leaving the interesting question as to the roles of the remaining three proteins? Characterisation of ICT1 as an integral component of the 55S mitoribosome has recently been published,³² but what are the functions of mtRF1 and c12orf65? In the absence of any identified proteins involved in rescue of aberrant translation or stalled hmt-ribosomes, a role for these proteins in a quality control capacity in translation and peptide release can be speculated.

What approaches can we use to investigate such issues pertinent to mitochondrial gene expression? Despite more than two decades of frustration, the field still lacks reliable and widely accepted methods of organellar transfection in intact cells and in vitro translation systems that would render us capable of asking more

direct and specific questions. Research into mammalian mitochondrial gene expression will benefit enormously once such tools have been developed. Currently, we must rely on cell lines defective in mt-translational, carrying defined mtDNA mutations in 'useful' positions, or on the imaginative design of experiments to help us elucidate the molecular mechanisms that govern human mitochondrial gene expression.

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