Supporting Online Material for

Hungry Codons Promote Frameshifting in Human Mitochondrial Ribosomes

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Published 15 January 2010, Science 327, 301 (2010)
DOI: 10.1126/science.1180674

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Hungry codons promote frameshifting in human mitochondrial ribosomes

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Abstract
Following the sequencing of human mtDNA almost 30 years ago, it has been generally accepted that AGA and AGG have been recoded as stop signals (S1). It was further assumed that an accommodation by mitochondrial release factor(s) must have occurred to ensure sequence specific recognition of these non-cognate stop codons (S2). Closer inspection of the mt-genome sequence shows that the AGA/AGG codons, which putatively terminate MTCO1 and MTND6 open reading frames are both immediately downstream of a U residue. These 2 codons do function in the terminal stage of translation but their primary role is to stall the mitoribosome, promoting a ribosomal frameshift that generates A-site UAG stop codons. In contrast to any current mitochondrial examples this frameshift would be in the –1 direction and not in the protein coding region. Thus, we show that human mitochondria are more conventional than previously supposed, and in line with most other mitochondrial systems, use only the more conservative UAA and UAG stop codons. Both codons are recognised by mtRF1a hence no modifications to release factors would need to have evolved.
Frameshifting in either direction has never been reported in the expression of mammalian mtDNA, although limited examples of classical +1 Programmed Ribosome Frameshifting (PRF) have been postulated or documented in other species. Studies have identified common single nucleotide insertions in the protein coding regions of a subset of mitochondrial genes from diverse species including oyster, glass sponge and ants (described in S3). All would require a similar +1 PRF for faithful production of the protein. It has led to the hypothesis that although rare, this +1 PRF mechanism must be tolerated in specific mitochondrial translation systems from a wide range of organisms (S3). The examples above described nucleic acid sequences that would predict the need for +1PRF to complete accurate mitochondrial protein synthesis. This contrasts with human mt-mRNA where all the coding sequences are known to be uninterrupted. However, we propose that human mitoribosomes do invoke –1 frameshift but at the stop codon after the proteins have been synthesised to completion, to allow recognition by a single mitochondrial release factor and facilitate release of the nascent polypeptide.

Is -1 ribosomal frameshifting supported by analysis of human mt-mRNA? Particular features that promote classical frameshifting have been well characterised in other translation systems. These include a ribosome that has paused or stalled as a consequence of either a rare or slowly decoded codon, an inhibitory secondary structure (S4-6) or an upstream heptapeptide ‘slippery sequence’ (S7, Fig S1). Precisely such paused mitoribosomes would be generated on human mt-mRNA by both AGA and AGG triplets, as no mt-tRNAs exist that recognize these codons. Further, these rare codons behave as ‘hungry codons’ in other translational systems under conditions of arginine deficiency, where they trigger a pause in translation and stimulate a –1 frameshift (S15-S16). In human mitochondria there are no cognate tRNAs to recognize AGA/AGG so these are ‘starving’ codons with no possibility of readthrough, which together with the downstream secondary structures that blocks forward movement, promote a frameshift in the -1 direction.

The upstream slippery sequence has been best characterised in viruses where the -1 frameshift is a commonly used mechanism. The heptanucleotide consensus is X XXY YYYZ (S7), however a large number of functional variations to this have been documented (described in S8). Since the mammalian 55S mitoribosome differs in a number of ways from the bacterial 70S and eukaryotic 80S counterparts (S9), the requirement for, position and the characteristics of a slippery sequence may be different, or it may not be required at all. In particular it is important to note that the A, P and E-site tRNAs are all reported to be involved in viral PRF (S10). However, no conventional E site is believed to exist in mitoribosomes (S9), reducing both the number and hence overall strength of codon/anticodon interactions. Since it is currently still not possible to transfect mammalian mitochondria the contribution from the flanking regions cannot be evaluated. With respect to the downstream element, it has been reported that such features act as positive modulators to significantly improve efficiency of poor heptanucleotide sequences (S11). Although human mtDNA is remarkably concise and the derived transcripts are often depicted as
lacking untranslated regions, although these are present in both \textit{MTCO1} and \textit{MTND6}; \textit{MTCO1} with the putative terminal AGA is followed by the antisense of tRNA\textsuperscript{ser} (UCN) that is \textit{(S12)}; similarly, the AGG of \textit{MTND6} is followed by UTRs of various lengths that extend into the antisense of \textit{MTND5} \textit{(S12)}. Folding algorithms \textit{(S13)} predict the antisense tRNA\textsuperscript{ser} (UCN) to generate a stable cloverleaf similar to a tRNA, and the sequence immediately downstream of \textit{MTND6} to form a stem loop (Fig S1). This bioinformatic data supports the hypothesis that \textit{MTCO1} and \textit{MTND6} transcripts contain features that can promote a -1 frameshift.

\textit{Can -1 frameshifting be demonstrated on human mtRNA?} We generated a mitochondrially targeted endoribonuclease, mtRelE, with sequence specificity that cleaves exclusively between nucleotides 2 and 3 of a codon in the ribosomal A-site. This allowed us to map \textit{in vivo} the exact stop codon used by \textit{MTCO1}. To initiate the fine mapping of the mtRelE cleavage site in mt-mRNAs we first analysed \textit{MTCO2} as it terminates in a preferred codon, UAG and would be predicted to lose its short (25 nt) 3’UTR on mtRelE cleavage. Tail length analysis indicated full-length, polyadenylated transcripts in wildtype cells (Fig. S3A lanes 1-4) consistent with normal oligo/polyadenylation \textit{in vivo} \textit{(S14)}. Post mtRelE induction a new population of shorter species became evident approximating to truncated but readenylated transcripts (Fig.S3A lanes 5-8). Sequencing (LM RT-PCR) confirmed specific removal of the 3’UTR followed by readenylation (Fig. S3A). Since \textit{MTCO2} uses UAG as a stop codon, cleavage between codon nucleotides 1/2 or 2/3 would generate UAAA\textsubscript{n}, making it impossible to identify the exact cleavage site. This same problem would exist for UAA codons, which constitute 9 of the 13 termination codons in human mitochondria. Therefore, to precisely map and resolve the issue of positional cleavage within the triplet we examined \textit{RNA14} the bicistronic unit with overlapping ORFs, reasoning that cleavage of the upstream \textit{MTATP8} UAG codon would release a stable downstream cleavage product. By mapping the 5’ termini of this species the precise mtRelE cleavage position would be revealed. Northern analysis indicated that post mtRelE induction greater than 90% of the remaining \textit{RNA14} was cleaved, resulting in mobility consistent with selective removal of \textit{MTATP8} (Fig. 1A). LM RT-PCR mediated cloning of the RNA species (Fig. S3B) demonstrated a limited number of full-length \textit{RNA14}, consistent with the Northern data. Crucially, however, all of the shortened species corresponded to specific mtRelE digestion at the preferred UAG termination codon, and cleavage only occurred between positions 2 and 3 (Fig. S3). These data confirmed that mitochondrially targeted RelE displays A-site specificity for UAG and UAA termination codons and cleaves only between nucleotides 2 and 3 of the codon. Taken together this suggests that the loss of protein synthesis of COX1 and presumably ND6 is a result of both open reading frames terminating in UAG and not AGA/AGG respectively (Fig.1). Final confirmation that \textit{MTCO1} uses UAG and not AGA as a termination codon came from \textit{MTCO1} sequences post-cleavage generated by LM RT-PCR (Fig.1). Although the newly formed codon:anticodon bonds would not be predicted, the sequence data indicates that these are tolerated in the human mitoribosome.
In conclusion, human mitochondria invoke -1 frameshifting. This is facilitated by mitoribosomes stalling at AGA/AGG codons for which there are no cognate mt-tRNAs, potentially in concert with diminished upstream codon/anticodon interactions due to a non-conventional E-site, and a secondary structure immediately downstream. In the absence of methods by which mammalian mitochondria can be transfected the contribution of these 5’ and 3’ elements cannot be interrogated. We have however, used a mitochondrially targeted endoribonuclease, mtRelE to confirm a mitoribosome shuffle that shunts the AGA/AGG from the A-site replacing them with the conventional stop codon, UAG. Thus, all 13 human mitochondrial open reading frames terminate in conventional UAA and UAG stop codons and can all be recognised by a single mitochondrial release factor. Perhaps mitoribosomal shuffling could also resolve the conundrum of internal translation initiation in the overlapping bicistronic transcripts?
Panel A depicts a composite cartoon of the characterised elements that promote PRF; an upstream slippery sequence (S7, S8), a rare ‘hungry’ codon (S15, S16) and a stable secondary structure downstream (S17). Permutations of these elements are present in mitochondrial transcripts *MTCO1* (B) and *MTND6* (C) (see text).
A cDNA containing the entire RelE ORF was prepared using primers RelE-preseq-For and RelE-Rev (Table S1) and pMG3323 as template (S23). The amplicon was BssHII/Not1 digested and ligated into pcDNA5/FRT/TO carrying the *N. crassa* ATP synthase subunit 9 presequence, generating and Su9-RelE fusion protein. Lysates (CL) and mitochondria (Mt) were prepared (S18) from human HEK293T cells (expressing mtRelE or wild type control). Mitochondria were proteinase K shaved and digitonin treated to reduce cytosolic and outer membrane contamination. To allow for organellar enrichment, 50µg CL sample was separated by 15 % PAGE compared to 10µg Mt. mtRelE was induced for 2 days (lanes 1-2) and compared with wild type untransfected cells (lanes 3-4). Western blot analysis was performed with antibodies to RelE; mtRF1a, another matrix located protein involved in translation; SDH 70 kDa member of complex II; and S6, a cytosolic ribosomal protein.

Fig. S2. *mtRelE is imported into the matrix of human mitochondria.*
Fig. S3. Expression of mtRelE results in specific cleavage of A-site codons in human mitochondrial ribosomes.

Cells expressing mtRelE show:- (A) stop codon selectivity, as shown here for MTCO2 where cleavage removes the short 3'UTR prior to readenylation (lower panel). (B) RNA14 sequence across the MTATP8/6 overlap indicates the respective stop and start codons. Post LM-PCR and cloning CACAT would precede the 5' terminal sequence of MTATP6, which upon cleavage by mtRelE between nucleotide position 2 and 3 would start GGCC. A representative electropherogram of RNA14 derived transcripts post mtRelE expression is shown. All of the mtRelE clones analysed indicated cleavage had occurred ONLY between 2nd and 3rd nucleotides (A and G).
Fig. S4. Schematic depicting methodology for 5’ and 3’ ligation mediated RT-PCR. RNA was prepared from isolated mitochondria and polynucleotide kinase treated prior to linker ligation. This was generally followed by standard RT-PCR and cloning into pPCRScript. Primers are given in Table S1.
Table S1. Oligonucleotide primers and linkers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Purpose</th>
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</thead>
<tbody>
<tr>
<td>RelE-preseq-For</td>
<td>CACACAGCGCGCTACTCTTCCATG GCGTATTTTCTGG</td>
<td>To generate pre-sequence and clone into pcDNA5/FRT/TO</td>
</tr>
<tr>
<td>RelE-Rev</td>
<td>ACTCGAGCGCCGCCGCTCGAATCGAGATGC</td>
<td>To clone into pcDNA5/FRT/TO</td>
</tr>
<tr>
<td>LIGN</td>
<td>PO₄⁻ ATGTGAGATCATGCACAGTCATA-NH₂</td>
<td>Linker for ligation to RNA 3’ termini</td>
</tr>
<tr>
<td>Anti-LIGN</td>
<td>GACTGTGCATGTCTCAC</td>
<td>Antisense to LIGN and 5’RNA LIGN for reverse transcription/PCR</td>
</tr>
<tr>
<td>5’RNA LIGN</td>
<td>HO-UAUGACUGUGCAUGAUCUCACAU-OH</td>
<td>RNA linker for ligation to RNA 5’ termini</td>
</tr>
<tr>
<td>MT41</td>
<td>GGTATACCTACGGTCAATG</td>
<td>Gene specific PCR primer for MTCO2</td>
</tr>
<tr>
<td>Hmt8265</td>
<td>TATAGCACCCCCCTCTACCCC</td>
<td>Radiolabeled primer for MTCO2 MPAT</td>
</tr>
<tr>
<td>Hmt7151</td>
<td>CATATTTCATCGCGTAAATC</td>
<td>Gene specific PCR primer for MTCO1</td>
</tr>
<tr>
<td>Hmt7410</td>
<td>CACACATTTCGAAGAAACCG</td>
<td>Gene specific PCR primer for MTCO1</td>
</tr>
<tr>
<td>Oligo 1</td>
<td>CTACCAACTCGAGAGATCT₃₀</td>
<td>Second round PCR with Hmt7410 to amplify polyadenylated sub-population of MTCO1</td>
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<tr>
<td>Hmt9206R</td>
<td>CACATGCAT₅₀TATGTGTTGTCGGTCAGG</td>
<td>Reverse transcription of RNA14</td>
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<tr>
<td>Hmt8669R</td>
<td>ACTTGTTGGGTGTTAGTC</td>
<td>Gene specific PCR primer for RNA14</td>
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<td>23Reverse</td>
<td>TAGGTCTGTTGTCGTAGGC</td>
<td>Reverse transcription of RNA7</td>
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<tr>
<td>22Reverse</td>
<td>ATGATTTGTCTGTCGGCTGTG</td>
<td>Gene specific PCR primer for RNA7</td>
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Materials and Methods

Tissue culture manipulations
Flp-In™T-Rex™-293 cells (HEK293T, Invitrogen) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10 % (v:v) FCS, 50 µg/ml uridine and 1 x non essential amino acids supplemented with 10 µg/ml Blasticidin S and 100 µg/ml Zeocin (Invitrogen). Post transfection selection was effected with Hygromycin B (100 µg/ml).

Stable transfection of HEK293T cells
Cells were transfected at ~50% confluency using Superfect (Qiagen) with the vectors pOG44 expressing FRT recombinase, and pcDNA5/FRT/TO containing sequence of the gene to be expressed (mtRelE with Su9 presequence) as previously described (S19). Primers are given in Table S1. Cells were induced with 1 µg/ml tetracycline to allow overexpression of mtRelE after one day transfection and finally harvested after 3 days.

Intramitochondrial localisation
Mitochondria were isolated from HEK293T cells expressing mtRelE and treated with DNase I (0.5 U/mg mitochondria; 15 min RT), proteinase K (5 µg/mg mitochondria; 30 min 4˚C) followed by 1mM PMSF inhibition, essentially as described (S19). Pelleted mitochondria were digitonin treated to remove the outer membrane (400 µg/mg mitochondria), washed and treated again with proteinase K (5 µg/mg mitochondria; 30 min 4˚C) followed by 1mM PMSF inhibition and washed twice prior to denaturation, separation through a 15% PAG and transfer to a PVDF membrane. Western blots were performed and developed as described (S18).

In vivo mitochondrial protein synthesis
Mitochondrial protein synthesis in cultured cells was performed as described (S20) after addition of emetine (100 µg/ml) and pulsed with [35S] methionine for 15 min. Gels were exposed to PhosphorImage cassettes and visualised with ImageQuant software. Proteins are identified by comparison against (S20).

siRNA constructs and transfection
siRNA specific to transcripts encoding mtRF1a (siRNA2, 5’-CCAUGACUGUAGCAUAUdTdT) was used to deplete the protein essentially as described in (S18). One day before transfection 2x10^5 Hek293T-WT and 3x10^5-mtRelE were seeded in 25 cm² flasks. Transfections were performed with Oligofectamine (Invitrogen) in Opti-MEM-I medium (Gibco) with final concentrations of 0.2 µM siRNA.

Determination of mRNA tail length (MPAT)
This method was described in detail in (S21). All relevant primers used in this study are given Table S1.

RNA isolation and northern blot analysis
RNA was prepared using TRIZOL (Invitrogen) following manufacturer’s recommendations. Northernns were performed as described (S22). Briefly, aliquots (3 µg) of RNA were electrophoresed through 1.2% agarose under denaturing conditions and transferred to GeneScreen
Plus membrane (NEN duPont) following manufacturer’s recommendations. Probes were generated from PCR products. Blots were probed with random hexamer labelled DNA fragments corresponding to internal regions of each gene, washed and visualised by PhosphorImage analysis.

**Ligation mediated RT-PCT and cloning**

All RNA was initially PNK treated to ensure ligatable ends post RelE cleavage. As depicted in Fig S4 linkers were ligated on to RNA as described in detail in (S2I).

Reverse transcription reactions (Superscript II, Invitrogen) were programmed with ~5 µg of RNA. Products were subjected to standard PCR, except for *MTCO1* where a seconds round of PCR was performed to select for polyadenylated species. PCR products were ligated into pPCRScript (Stratagene), and transfected into bacterial strain DH5α. DNA was PCR amplified directly from colonies lysed with Triton X-100, using primers designed to regions spanning the plasmid cloning site. These products were purified using QIAquick gel purification column (Qiagen) prior to DNA sequencing using BigDye terminator cycle sequencing chemistry (PE Biosystems) on an ABI 377 automated DNA sequencer.
References

S2. L. Kisselev, M. Ehrenberg, L. Frolova, EMBO J 22, 175 (2003).

S24. This work was supported by the Wellcome Trust [074454/Z/04/Z] and Biotechnology and Biological Sciences Research Council [BB/F011520/1]. We thank Kenn Gerdes for kindly providing the clone and antibodies to bacterial RelE.