Hungry codons promote frameshifting in human mitochondrial ribosomes

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\textit{One sentence summary}
Ribosomal frameshifting has not been reported to occur in human mitochondria, however, by the use of a bacterial endoribonuclease we show that this does indeed occur negating the apparent reassignment of AGA/AGG as stop codons.
Abstract

Human mitochondria are not strict adherents to the universal genetic code with modifications that include the apparent recoding of 2 arginine triplets to termination signals. This use of both AGA and AGG occurs rarely in other mammals and the precise mechanism that has driven the change from encoding arginine to dictating a translational stop has posed a challenging conundrum. Sequence data reveals that a –1 frameshift of the mitoribosome upstream of the rare codons would necessitate the recognition of only the conventional UAA and UAG termination codons. With the use of a sequence specific endoribonuclease we show that the rare arginine codons presumably in association with other cis-elements do indeed promote frameshifting in human mitoribosomes.

Following the sequencing of human mtDNA, it has been generally accepted that AGA and AGG have been recoded as stop signals (1). 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 (2). Closer inspection of the mt-genome sequence shows that the AGA/AGG codons that are predicted to terminate the 2 open reading frames, *MTCO1* and *MTND6* respectively, are both immediately downstream of a U residue. It is therefore possible that although these 2 codons do function in the terminal stage of translation, this role is primarily to stall the mitoribosome, causing a ribosomal frameshift on the human mt-mRNA. In contrast to any current mitochondrial examples this frameshift would be in the –1 direction and not in the protein coding region. Further, no modifications to release factors would need to have evolved.

As a consequence of a -1 ribosomal frameshift, both of the open reading frames (ORFs) would terminate in the standard UAG codon and thereby follow the same ‘2 stop codon’ (UAA, UAG) mechanism common to many other mitochondrial gene expression systems (3). 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. The ostrich
nad3 gene, for example, was reported to encode only a truncated protein (4), but after examining sequences from a number of bird and turtle species, Mindell et al (5) noticed that only a single base insertion was responsible for the interruption of the ORF, resulting in a premature stop. It was, therefore, postulated that mitochondrial translation must invoke a +1 PRF. More recently, other 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 6). 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 (6). 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 the 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. One of the most commonly conserved is a ribosome that has paused or stalled as a consequence of either a rare or slowly decoded codon, or an inhibitory secondary structure (7-9). 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 identical rare codons behave as ‘hungry codons’ in other translational systems under conditions of arginine deficiency, triggering a pause in translation and stimulating a –1 frameshift (8, 9).

Two further common features that promote -1 frameshifting are an upstream heptapeptide ‘slippery sequence’ and a downstream sequence that can generate a stable secondary structure such as a stem loop or pseudoknot (7). These features in the context of human mitochondrial MTCO1 and MTND6 transcripts are discussed below (Fig 1).
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 YYZ (10), however a large number of functional variations to this have been documented (described in 11). Since the mammalian 55S mitoribosome differs in a number of ways from the bacterial 70S and eukaryotic 80S counterparts (12), the requirement for, position and the characteristics of a slippery sequence may be different. In particular it is important to note that the A, P and E-site tRNAs are all reported to be involved in viral PRF (13). However, no conventional E site is believed to exist in mitoribosomes (12), reducing both the number and hence overall strength of codon/anticodon interactions. Further, as human mitochondrial translation uses only 22 mt-tRNAs to recognize 60 codons, mt-tRNAs generate a looser codon:anticodon interaction than other systems by using a “2 out of 3” base interaction (14). Finally, when the A-site is occupied by an AGA or AGG codon there will be no corresponding mt-tRNA. Therefore, in contrast with conventional -1PRF, only a weak mt-mRNA(mt-tRNA interaction will need to be broken to allow reformation in the -1 frame.

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 (15). Although human mtDNA is remarkably concise and the derived transcripts are often depicted as having no untranslated regions, this is not entirely correct. In the case of MTCO1 the putative terminal AGA is followed by the antisense of tRNA\text{ser}^{\text{UCN}} that is generally not cleaved on processing (16). Similarly, the AGG of MTND6 is followed by UTRs of various lengths that extend into the antisense of MTND5 (16). Using folding algorithms (17), the antisense tRNA\text{ser}^{\text{UCN}} is predicted to generate a stable cloverleaf similar to a tRNA, and the sequence immediately downstream of MTND6 is predicted to form a stem loop (Fig 1). This bioinformatic data supports the hypothesis that MTCO1 and MTND6 transcripts contain features that can promote a -1 frameshift.
Can -1 frameshifting be demonstrated on human mtRNA? To experimentally determine whether -1 frameshifting occurs in human mitochondria, we attempted to fine map the precise sequence of the \textit{MTCO1} termination codon. For this we constructed a mitochondrially targeted version of RelE (mtRelE, \textit{18}), an endoribonuclease that has previously been shown to specifically cleave RNA occupying the A site of the bacterial ribosome. Further, this enzyme shows marked sequence preference for the standard termination codons UAG and UAA. Human HEK293T cells were prepared to inducibly express mtRelE (\textit{18}), which was efficiently localised and imported into the mitochondrial matrix as evidenced by protection from protease digestion after removal of the outer mitochondrial membrane (Fig S1).

RelE has very distinct sequence selectivity; UAG is the preferred substrate, followed by UAA, with negligible predicted recognition of AGA/AGG and non detectable K\textsubscript{cat}/K\textsubscript{m} values for AGA (\textit{18}). This would infer that production of COX1 and ND6 whose ORFs putatively terminate in AGA/AGG should be unaffected. Global effects on mitochondrial translation were, therefore, assessed by metabolic labelling (\textit{35}S-met) of wildtype and mtRelE induced cells (\textit{19}). Strikingly, however, \textit{de novo} synthesis of most mt-proteins including COX1 and ND6 was robustly reduced (Fig2A). Northern analysis of control cells post mtRelE expression revealed the majority of \textit{MTCO1} (68 ± 1.73 %, n=3) and \textit{MTCO2} (70 ± 1.4 %, n=3) transcripts to be intact at steady state (Fig2B lanes 1 and 3). Hence, the loss of \textit{de novo} protein synthesis could not be attributed to non-specific degradation of transcripts by mtRelE. Depletion of the endogenous mitochondrial release factor mtRF1a, is known to stabilise transcripts possibly through an extended association of the mt-mRNA with the mitoribosome. RelE expression promotes release of cleaved mRNA from the bacterial ribosome (\textit{18}). Therefore, mtRelE expression would be predicted to release cleaved transcripts from the mitoribosome abrogating mitoribosome mediated protection. As shown in Fig2B mtRF1a depletion in tandem with mtRelE expression does indeed relieve such protection of all mt-transcripts analysed and markedly so for \textit{MTCO1} (Fig2B lanes 2 and 4) where the steady state level is reduced post mtRelE expression, indicating both recognition and cleavage by mtRelE. Northern analysis, could not resolve whether the
short 3’ UTRs present in MTCO1 (72 nt) had been lost post mtRelE cleavage. MTND5, however, possesses a more extensive 3’ UTR (568nt). On mtRelE induction, a species was detected with increased mobility consistent with cleavage at the stop codon and loss of this 3’UTR (Fig2B lanes 3-4 indicated by *). Human mtDNA encodes 2 transcripts with overlapping open reading frames encoding MTATP8/6 (RNA14) and MTND4L/4 (RNA7). Cleavage at the stop codon of the upstream ORF would release an mRNA with a 5’-truncated downstream ORF. As with MTND5, novel species were detected on mtRelE expression. This was particularly apparent for RNA14 where MTATP8 terminates in UAG, the preferred stop codon for RelE (Fig2B).

To initiate the fine mapping of the mtRelE cleavage site in mt-mRNAs we first analysed MTCO2 as it terminates in the preferred UAG and would be predicted to lose its short (25 nt) 3’UTR on mtRelE cleavage. Analysis by tail length assay indicated full-length polyadenylated transcripts in wildtype cells (Fig2C lanes 1-4). Processing of L-strand transcript 3’ termini in vivo normally leads to oligo- or polyadenylation (20). Consistent with this, a second population of shorter species of a length approximating to readenylated truncated transcripts, became evident post mtRelE induction (Fig2C lanes 5-8). To reveal the cleavage site, truncated species were sequenced after ligation mediated RT-PCR (LM RT-PCR, 19) and subsequent cloning. All sequences confirmed specific removal of the 3’UTR followed by readenylation (Fig2C). However, since MTCO2 uses UAG as a stop codon, cleavage between either positions 1/2 or 2/3 within the codon would generate UAAA₃, making it impossible to identify whether cleavage had occurred after nucleotide 1 or 2. The same problem would be encountered with the UAA codons, which constitute 9 of the 13 termination codons in human mitochondria.

To resolve the issue of positional cleavage within the triplet we examined RNA14 the bicistronic unit with overlapping ORFs, reasoning that cleavage of the upstream 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 MTRNA14...
was cleaved, resulting in mobility consistent with selective removal of *MTATP8* (Fig2B). LM RT-PCR mediated cloning of the RNA species (FigS2) demonstrated a limited number of full-length *RNA14*, consistent with the Northern data. Crucially, however, all of the shortened species corresponded to specific mtRelE digestion at the UAG termination codon and cleavage only occurred between positions 2 and 3 (Fig3).

Mitochondrially targeted RelE, therefore, 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. However, to finally confirm that *MTCO1* uses UAG and not AGA as a termination codon, we cloned and sequenced LM RT-PCR generated products of *MTCO1* post-cleavage. As mtRelE cleaves only between the 2\(^{nd}\) and 3\(^{rd}\) nucleotides of the stop codon, a prediction can be made following mtRelE expression; use of AGA as termination codon would result in –AAAAUCUAG\(_n\) whilst UAG would produce –AAAAUCUAA\(_n\). RNA from control cells was analysed, 10 of the 12 clones reflected the predicted full-length *MTCO1* transcript containing the 3’UTR, the remaining 2 were partial truncations in the antisense tRNA\(^{\text{Ser}}\), commonly identified in the EST database. This species was also found in 2 of the mtRelE samples. All of the remaining 33 mtRelE clones however, terminated in -AAAAUCUA followed by readenylation (Fig3B), signifying that *MTCO1* terminates at UAG rather than AGA.

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. Perhaps
mitoribosomal shuffling could resolve the conundrum of translation initiation in the overlapping bicistronic transcripts?

References
17. http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi and empirical.
19. Materials and methods are available as supporting material on Science Online.
21. 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.
Figure legends

Fig.1. Features that promote –1 frameshifting.
Panel A depicts a cartoon of the characterised elements that promote PRF; an upstream slippery sequence (10, 11), a rare ‘hungry’ codon (8, 9) and a stable secondary structure downstream (12). Variations on these elements are present in mitochondrial transcripts MTCO1 (B) and MTND6 (C) (see text).

Fig.2. Expression of mtRelE results in specific cleavage of mt-mRNA stop codons.
Cells expressing mtRelE show:- (A) reduced metabolic labelling of mtDNA encoded gene products ; (B) specific cleavage of mt-mRNA generating novel products depicted by * in both wild type cells and those treated with siRNA to mtRF1a ; and (C) stop codon selectivity, as shown here for MTCO2 where cleavage removes the short 3’UTR prior to readenylation (lower panel).

Fig.3. Sequence analysis of RNA14 and MTCO1 transcripts post mtRelE expression.
A. 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).
B. Representative electropherogram of MTCO1 derived transcripts from wild type (WT) cells and post mtRelE expression. Sequences are given below with the mtRelE cleavage site designated by *. WT has retained the 3’UTR whilst 33/35 mtRelE demonstrated loss of the 3’UTR (lower panel), consistent with cleavage at the UAG stop codon. The remaining 2 clones showed truncated WT sequences identified in EST databases.