Functional polypeptides can be synthesized from human mitochondrial transcripts lacking termination codons

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The human mitochondrial genome (mtDNA) is a small, circular DNA duplex found in multi-copy in the mitochondrial matrix. It is almost fully transcribed from both strands to produce large polycistronic RNA units that are processed and matured. The 13 mtDNA-encoded polypeptides are translated from mt-mRNAs that have been matured by polyadenylation of their free 3'-termini. A patient with clinical features consistent with an mtDNA disorder was recently shown to carry a microdeletion, resulting in the loss of the termination codon for *MTATP6* and in its juxtaposition with *MTCO3*. Cell lines from this patient exhibited low steady-state levels of *RNA14*, the bi-cistronic transcript encoding subunits 6 and 8 of the F₀F₁-ATP synthase, complex V, consistent with a decreased stability. Recent reports of 'non-stop' mRNA decay systems in the cytosol have failed to determine the fate of gene products derived from transcripts lacking termination codons, although enhanced decay clearly required the 'non-stop' tran-

scripts to be translated. We wished to determine whether functional translation products could still be expressed from non-stop transcripts in the human mitochondrion. Although a minor defect in complex V assembly was noted in the patient-derived cell lines, the steady-state level of ATPase 6 was similar to controls, consistent with the pattern of *de novo* mitochondrial protein synthesis. Moreover, no significant difference in ATP synthase activity could be detected. We conclude that, in the absence of a functional termination codon, although mitochondrial transcripts are more rapidly degraded, they are also translated to generate stable polypeptides that are successfully integrated into functional enzyme complexes.

Key words: mitochondrial gene expression, mitochondrial translation, mtDNA (mitochondrial DNA) disease, mt-mRNA polyadenylation, non-stop translation, termination codon.

INTRODUCTION

The mitochondrial genome (mtDNA) is located in the mitochondrial matrix of all human nucleated cells. It encodes 22 mt-tRNAs, two mt-rRNAs and 13 polypeptides, all of which are believed to be essential components of the complexes that couple oxidative phosphorylation. This relatively small genome (approx. 16.5 kb [1]) is almost fully transcribed from both strands to produce polycistronic RNA units that are processed and matured. Human mtDNA has evolved into a remarkably compact genome, with limited non-coding material, no introns and with truncated termination codons that often require polyadenylation for completion [2]. The polycistronic units are punctuated by mt-tRNAs that are processed, in part, by a mitochondrial RNase P [3] and are matured with a 3'-terminal-CCA addition by a bacterial-type nucleotidyltransferase [4]. Mitochondrial mRNAs are adenylated at their 3'-termini by the addition of 50–60 residues [5,6], but the enzyme(s) responsible is currently unknown. Further, it is not clear whether the extension occurs progressively or a poly(A)⁺ (polyadenylated) unit is added *en bloc*. There is no obvious candidate for a mitochondrial poly(A)⁺ polymerase or polynucleotide polymerase in the human genome, suggesting that there might be differentially spliced isoforms of both or either of these genes that could encode mitochondrially destined polypeptides or that a subpopulation of protein is localized to the mitochondrion [7].

mtDNA is present in many copies per cell and mutations are known to cause a series of chronic progressive disorders [8,9]. As mtDNA encodes proteins that are essential for aerobic respiration,

it is possible that any tissue or cell in the body could be affected. Although mtDNA mutations can indeed cause a wide variety of tissue defects, the most commonly affected tissues are muscle and the central nervous system [10]. Discrete mtDNA lesions are often associated with specific syndromes which, in themselves, are rare. Taken together, however, mtDNA mutations comprise an important group of heritable disorders. A recent study in the northeast of England revealed that as many as 1:8000 individuals carry a pathogenic mtDNA mutation and 1:17 000 show clinical defects associated with mtDNA disease [11].

To gain further insight into the molecular aetiology of these diseases and to increase our understanding of mitochondrial gene expression, we have been investigating cell lines derived from patients with disorders of the mitochondrial genome. One such cell line was derived from a patient who presented as a child with repetitive bouts of lactic acidemia and other symptoms consistent with mtDNA disease [6,12]. The initial study reported that the child had an apparently homoplasmic (present in all mtDNA copies) 2 bp microdeletion of mtDNA ($\mu\Delta$), which removed the two residues at or between the ends of *MTATP6* and the *MTCO3* gene. In the wild-type, the 3'-terminal uridine of this transcript, after polyadenylation, forms the stop codon for *RNA14*, the bi-cistronic RNA unit in part encoded by *MTATP6* [3' U plus 5' PO₄ -A-A(n)-3' creates a UAA termination codon]. Further, assuming that the normal RNA processing event between *RNA14* and *RNA15* (*MTCO3* transcript encoding COX3 protein, where COX stands for cytochrome *c* oxidase) is lost as a consequence of the deletion, this juxtaposition is predicted to result in an ATPase 6–COX3 in-frame fusion protein. In

Abbreviations used: BN, Blue native PAGE; COX, cytochrome *c* oxidase; CRS, Cambridge Reference Sequence; eRF1, eukaryotic peptide chain release factor 1; FCS, foetal calf serum; mtDNA, mitochondrial DNA; poly(A)⁺, polyadenylated; RF, release factor; $\mu\Delta$, microdeletion cell line.

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patient-derived fibroblasts, however, we were able to show that the bi-cistronic RNA that encodes ATPases 8 and 6 ($\mu\Delta RNA14$) could still be cleaved successfully from *RNA15*, despite the loss of the wild-type processing site [6]. Polyadenylation of $\mu\Delta RNA14$ was retained, but it was rapidly degraded following translation, resulting in markedly decreased steady-state levels. Polyadenylation of the 3'-terminus of $\mu\Delta RNA14$ results in a transcript lacking a natural termination codon, which would be predicted to generate a translation product (ATPase 6) having multiple lysine residues at the C-terminus. Crucially, it is also not clear how translation would terminate, how the terminal peptidyl tRNA bond could be cleaved, how the mitoribosomal complex would dissociate and, indeed, what is the fate of the translation product?

Surveillance mechanisms do exist to remove aberrant transcripts such as those missing or containing inappropriate termination codons. These include the non-stop [13] and non-sense-mediated decay pathways [14], both of which operate in the eukaryote cytosol. Moreover, eubacteria also maintain a mechanism, involving a tmRNA molecule for removing ribosomes that have stalled on transcripts, often as a consequence of absent stop codons [15]. Although it is clear that non-stop transcripts such as $\mu\Delta RNA14$ are rapidly degraded in the mitochondrion after translation, the fate or efficacy of the translation products has not been determined. In situations where it has been possible to study the translation of non-stop mRNAs in other systems, the resultant polypeptides are either tagged for rapid degradation (as in eubacteria) or cannot be found, consistent again with rapid turnover [16]. Thus the aim of the present study was to determine whether a mitochondrial transcript lacking a termination codon could be translated and, in contrast with examples from other species or cellular locations, whether the resultant polypeptides were stable and functional.

EXPERIMENTAL

Tissue culture conditions

Fibroblasts were cultured in Eagle's minimal essential medium supplemented with 10 % (v/v) FCS (foetal calf serum), non-essential amino acids, 2 mM L-glutamine, 1 mM pyruvate and 50 μ g/ml uridine at 37 °C under humidified, 5 % CO₂. All reagents were purchased from Sigma and plastic ware from Corning Costar (Corning BV Life Sciences, Schiphol-Rijk, The Netherlands).

DNA analysis

DNA was isolated from fibroblasts by phenol/chloroform extraction and ethanol precipitation after overnight incubation at 37 °C in 1 % (w/v) SDS and proteinase K (2 mg/ml final concentration). Whole genome sequencing was performed on PCR products spanning the entire genome using the primers detailed in [17].

Heteroplasmy was determined by restriction-fragment-length polymorphism analysis. PCR amplification of mtDNA (corresponding to nt 9183–9329 [1]) was achieved using mismatched primers 5'-CCTCTACCTGCACGACAATGCA-3' and 5'-CGTTATGCATTGGAAGTGAAATCAC-3' (mismatch bases indicated in boldface). Reaction conditions (50 μ l) were 1 $\times$ reaction buffer IV (AB Gene, Epsom, Surrey, U.K.), 1.5 mM MgCl₂, 200 μ M dNTPs, 50 pmol of each primer, 2 units of *Taq* DNA polymerase (AB Gene) and 100 ng of template DNA with the following profile: 1 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 62 °C, 1 min at 72 °C, followed by a final 7 min extension at 72 °C. Re-

actions were then supplemented with 9.8 pmol of each primer, 1 unit of *Taq* DNA polymerase and 4 μ Ci of [α -³²P]dCTP (3000 Ci/mmol; Amersham Biosciences, Little Chalfont, Bucks., U.K.) and subjected to a final cycle of 4 min at 95 °C, 2 min at 62 °C and 10 min at 72 °C.

For restriction-fragment-length polymorphism analysis, the radiolabelled 147 bp amplicon was digested with *Nsi*I that cuts the control sample into fragments of approx. 116, 22 and 9 bp, whereas $\mu\Delta 9205$ cuts only once, generating fragments of 138 and 9 bp. These fragments were resolved on a 15 % (w/v) non-denaturing polyacrylamide gel and visualized by PhosphorImager analysis (Amersham Biosciences).

SDS/PAGE and Western-blot analysis

Pellets of mitochondrially enriched fractions, prepared as for Blue native gels (see below), were resuspended in PBS. Bradford protein estimations were performed and 20 μ g of each sample was dissociated [10 % (v/v) glycerol, 125 mM Tris (pH 6.8), 2 % (w/v) SDS and 50 mM dithiothreitol] and separated by SDS/PAGE (15 % gel). Transfer on to a PVDF membrane (Millipore) was effected in 25 mM Tris/HCl, 192 mM glycine, 0.02 % (w/v) SDS and 15 % (v/v) methanol for 1 h at 100 V. All membranes were blocked in 5 % (w/v) milk TTBS [20 mM Tris, pH 7.5/0.5 M NaCl/0.05 % (v/v) Tween 20] for 1 h before incubation with primary antisera, also in milk TTBS. Blots were washed three times for 5 min with TTBS before and after incubation with secondary antibodies and visualized using ECL[®]-plus (Amersham Biosciences).

³⁵S-metabolic labelling of mitochondrial proteins

Protein translations were performed essentially as described in [18]. Cells were cultured in 35 mm dishes to approx. 60 % confluency. The medium was removed and cells were rinsed twice for 5 min in methionine-free, FCS-free Dulbecco's modified Eagle's medium. Cells were then incubated in methionine-free Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) dialysed FCS, 10 μ g/ml emetine and 500 μ Ci [³⁵S]methionine (1000 Ci/mmol; Amersham Biosciences) and returned to the 37 °C, 5 % CO₂ incubator for 1 h. After removal of the incubation medium, cells were washed with PBS and a cell pellet was prepared. Protein samples containing 50 000 d.p.m. were separated by SDS/PAGE (15 % gel). The products were visualized by PhosphorImager analysis (Amersham Biosciences).

Analysis of F₀F₁-ATP synthase

Mitochondrially enriched fractions and Blue native gels were prepared exactly as described in [19]. Briefly, controls and patient fibroblasts (1 $\times$ 10⁶ cells/sample) were harvested, washed with PBS and permeabilized with digitonin (4 mg/ml final concentration) at 4 °C for 10 min. Cold PBS (5 vol.) was added, followed by centrifugation (at 10 000 g for 5 min at 4 °C); pellets were washed with PBS and then processed for BN (Blue native PAGE). Samples were electrophoresed through a first dimension consisting of a continuous 5–15 % acrylamide gel, followed by a second dimension consisting 10 % (w/v) Tris/Tricine SDS/polyacrylamide gel, both with 4 % stacking gels. Transfer on to nitrocellulose membranes was performed in Towbin buffer [25 mM Tris, pH 8.5/192 mM glycine/20 % (v/v) methanol/0.1 % (v/v) SDS] for 1 h at 100 V.

Table 1 Position of $\mu\Delta$ and previously unreported changes from revised CRS

Change from revised CRS	Gene	Change
DEL 9205/6	<i>MTATP6</i>	
C9335T	<i>MTCO3</i>	Silent
A11362G	<i>MTND4</i>	Silent
A12822G	<i>MTND5</i>	Silent
T15287C	<i>MTCYTB</i>	Phe to Leu
T15705C	<i>MTCYTB</i>	Leu to Pro

ATP synthesis measurements

Assays were performed on digitonin-permeabilized cells essentially as described by Wanders et al. [20]. Optimal digitonin concentration was initially determined by assaying phosphoglucose isomerase activity and by subsequent assays performed with cells permeabilized by 20 and 40 $\mu\text{g/ml}$ digitonin. ATP synthesis was measured at 37 °C for 20 min using 100 mM glutamate and 10 mM malate as the respiratory substrate; reactions were terminated by the addition of HClO_4 and neutralized by KOH. ATP synthesis was then measured in these protein-free extracts as described previously [21]. Assays were also performed in the presence of the complex V inhibitor oligomycin (at 20 $\mu\text{g/ml}$) to demonstrate specificity.

RESULTS

mtDNA analysis of $\mu\Delta 9205$

Original analysis of the patient by DNA sequencing of part of the mitochondrial genome and PCR-SSCP (single-strand conformation polymorphism) of the *MTATP6* region reported that both fibroblasts and peripheral blood lymphocytes appeared to be homoplasmic for the microdeletion [12]. In the present study, we performed DNA sequencing of the complete mitochondrial genome and definitively assessed the microdeletion load in $\mu\Delta$. The genome contained 42 changes from the revised CRS (Cambridge Reference Sequence) [22], including $\mu\Delta 9205$, and was consistent with the genotype being from the rare haplogroup I [23]. There were five replacements that had not been reported previously (<http://www.mitomap.org>), three of which were neutral substitutions: the two remaining non-synonymous substitutions were in *MTCYB* (Table 1).

Our sequencing of an amplicon covering the *MTATP6*/*MTCO3* region also failed to provide evidence of wild-type molecules in this patient-derived fibroblast line. To determine more accurately any potential level of heteroplasmy of the microdeletion in the patient-derived cell line, DNA was isolated from various passages and last cycle hot PCR was performed. As can be seen (Figure 1), this mutation was present in levels > 98 % of the total mtDNA population. Further, Southern-blot analysis confirmed that the relative levels of nuclear DNA and mtDNA are similar in control and $\mu\Delta$ cells (results not shown).

Steady-state and *de novo* syntheses of ATPase 6 are unaffected in the patient-derived cell line

Northern-blot analysis had revealed previously that the steady-state level of the bi-cistronic RNA unit encoding ATPase 6 (*RNA14*) was significantly decreased in the $\mu\Delta$ cell line [6]. A substantial amount of $\mu\Delta\text{RNA14}$ had poly(A) tails shortened as

Figure 1 Determination of the levels of heteroplasmy for $\mu\Delta 9205$

DNA was prepared from control (Cont) and patient ($\mu\Delta$) fibroblasts and a 147 bp amplicon spanning the mutation was generated with mismatched primers as detailed in the Experimental section. After radiolabelling the amplicon in the last cycle, an *Nsi*I digestion was performed, resulting in fragments of approx. 116, 22 and 9 bp in the control compared with 138 and 9 bp for the $\mu\Delta$. Products were separated on a non-denaturing polyacrylamide gel and visualized by PhosphorImage analysis using ImageQuant software. mtDNA from patient $\mu\Delta$ appears homoplasmic for $\mu\Delta 9205$, but owing to the resolution of the system, we can only be confident that the patient cell line carries a minimum of 98 % $\mu\Delta 9205$.

a consequence of translation. *RNA15*, however, was unaffected. To determine whether the lowered level of $\mu\Delta\text{RNA14}$ caused a decrease in the steady-state level of ATPase 6, we isolated a mitochondrially enriched fraction and subjected it to Western-blot analysis. When the $\mu\Delta$ fraction is compared with the two control lines (Figure 2A), the relative levels of ATPase 6 are similar, as are those of COX2, another mitochondrially encoded subunit of the respiratory chain, in addition to crotonase and glutamate dehydrogenase, both nuclear-encoded but mitochondrially destined proteins.

To study the *de novo* synthesis of ATPase 6, *in vivo* metabolic labelling of mitochondrial polypeptides was performed. As shown in Figure 2(B), *in vivo* mitochondrial protein synthesis of fibroblasts from $\mu\Delta$ revealed a pattern of products similar to the control cell line. This experiment measured the overall amount of [^{35}S]methionine incorporated into mitochondrial products over a single time point (1 h) and does not indicate a synthesis rate. Assays were performed on seven separate occasions, with samples electrophoresed through a variety of gradient and linear SDS/polyacrylamide gels. The migration of the COX2, COX3 and ATPase 6 triplet was observed to vary between gels, potentially as a consequence of minor alterations in buffer pH, as has been documented previously [18]. The difference seen in ND4L/ATPase 8 was not observed on any other occasion and, therefore, it was not considered representative of the $\mu\Delta$ cell line. There was no conclusive evidence of decreased amounts of ATPase 6 protein or of a difference in mass of the polypeptide as a putative consequence of additional lysine residues.

Figure 2 Steady-state and *de novo* syntheses of ATPase 6 in the $\mu\Delta$ cell line

(A) Mitochondrially enriched fractions (20 μ g) from two independent fibroblast controls and the $\mu\Delta$ patient were separated by PAGE (15 % gel) and subjected to Western-blot analysis as detailed in the Experimental section. Levels of ATPase 6 were comparable with controls as were the steady-state levels of the mitochondrially encoded COX2 and the nuclear-encoded mitochondrial proteins glutamate dehydrogenase (GDH) and crotonase. Even through this high-percentage polyacrylamide gel, the migration of ATPase 6 in patient and control mitochondria appeared to be identical. (B) Fibroblasts (control and $\mu\Delta$) were subjected to *de novo* mitochondrial protein synthesis in the presence of [35 S]methionine for 1 h after the poisoning of cytosolic protein synthesis with emetine. Cells were lysed, aliquots of protein corresponding to equal numbers of counts (50 000 d.p.m.) separated by SDS/PAGE (15 % gel) and radiolabelled proteins were visualized by PhosphorImage analysis. After minor alterations in the gel running conditions, it was possible to resolve ATPase 6 as shown. No significant difference in the amount or mass of this product could be detected between the two cell lines. Positions of molecular-mass standards (kDa) are indicated to the right of the gel and mitochondrial products are identified by comparison with [18]. On this particular image, a stronger signal was noted for the short products running close to the dye front (labelled ND4L/ATPase 8) in $\mu\Delta$. This was a minor artifact and was not noted on any other similar analysis.

The original report predicted that the microdeletion would destroy normal processing, resulting in increased levels of an RNA14/15 tri-cistronic RNA unit, which when translated would produce an in-frame fusion protein of ATPase 6 and COX3. Although such an uncleaved transcript was detected in the microdeletion patient, it is at similar levels to that observed in control lines [6]. Our metabolic labelling studies were unable to detect any fusion protein.

$\mu\Delta$ has a minor defect in complex V assembly

Western-blot analysis and *in vitro* synthesis results (Figures 2A and 2B) demonstrate that $\mu\Delta$ RNA14 can still be translated despite the observation that all the 3'-termini of $\mu\Delta$ RNA14 lacked a termination codon [6]. The poly(A) extension would be predicted to be translated as polylysine. Although the natural length of the poly(A) extension is truncated in the $\mu\Delta$ transcripts, the short poly(A) would still be predicted to add several lysine residues to the C-terminus of ATPase 6, and it is not clear how the translating ribosome would disaggregate from the nascent polypeptide. Therefore it is possible that if the ATPase 6 is synthesized with this short extension that might not be identifiable by standard gel-electrophoresis techniques, the aberrant protein could affect normal assembly of the F_0F_1 -ATP synthase, complex V. To analyse complex assembly, a mitochondrially enriched fraction was subjected to BN. BN is a technique designed to allow the separation of complexes without causing their dissociation. Judicious use of detergents has allowed this technique to be applied successfully to components of the mitochondrial respiratory chain, effecting their release from the inner mitochondrial membrane [19,24]. Samples prepared from a patient and

two different fibroblast control lines were subjected to BN, and Western-blot analysis was performed on the second dimension (Figure 3). Antisera against the α -subunit of the F_1 -ATPase demonstrated levels of fully assembled complex V similar to that of the controls, with somewhat increased levels of free F_1 and marginally higher levels of unincorporated α -subunit. In-gel activity assays for F_1 -ATPase were performed on patient samples electrophoresed through the first dimension only, utilizing the histochemical stain for ATP hydrolysis (results not shown). Free F_1 was observed in the patient-derived mitochondria on both the immuno- and histochemical analyses. This may reflect a greater susceptibility of the assembled complex V in the $\mu\Delta$ patient to disruption under the conditions used for BN separation, possibly as a consequence of a very short lysine extension.

Similar rates of ATP synthesis are observed in control and $\mu\Delta$ cells

There is no evidence that $\mu\Delta$ 9205 causes any effect on the synthesis or stability of ATPase 6 or a major change in its integration into the F_0F_1 -ATP synthase. It is possible, however, that if the microdeletion promotes the addition of a short polylysine extension, the integrated product could still affect the function of complex V. To assess this, rates of ATP synthesis were measured in digitonin-solubilized cells (Figure 4) using glutamate and malate as the respiratory substrate, with control lines demonstrating rates comparable with those reported previously [25]. This was significantly decreased in the presence of the F_1 -ATPase inhibitor oligomycin (at 20 μ g/ml), in agreement with previous observations [20]. However, as shown in Figure 4, the activity was not significantly affected in $\mu\Delta$ cells when compared with control fibroblast lines.

Figure 3 F_0F_1 -ATP synthase assembly in $\mu\Delta$ and control fibroblast cell lines

BN was performed on mitochondrially enriched samples from $\mu\Delta$ and two independent control cell lines and is presented in the three panels of the Figure. The first dimension separates intact complexes, which are then dissociated in the second dimension into individual subunits. Anti- F_1 α -subunit antibodies reveal the full F_0F_1 -ATP synthase complex and, where present, the assembled F_1 alone and the unassembled α -subunit. In each extract, fully assembled F_0F_1 -ATP synthase was observed, but only in the $\mu\Delta$ sample there was evidence of isolated F_1 . Anti-COX2 antibodies highlight the COX2 subunit, a member of the mitochondrial respiratory enzyme COX (complex IV).

DISCUSSION

Analysis of fibroblasts from patient $\mu\Delta$ has provided further insight into the resilience of the mitochondrion in maintaining a normal function, despite harbouring an mtDNA mutation that causes aberrant mitochondrial gene expression. Sequencing of the entire genome revealed a rare haplogroup I with some previously undocumented silent polymorphisms, in addition to the essentially homoplasmic $\mu\Delta 9205$, and two non-synonymous novel substitutions in *MTCYB*. It was not our aim to determine the exact molecular aetiology underlying the clinical disorder for patient $\mu\Delta$ and the present study cannot formally discount a pathogenic mutation in this cell line being in *MTCYB*. However, aberrant RNA metabolism of *RNA14* as a consequence of the microdeletion has already been established and the focus of this investigation was to identify whether $\mu\Delta RNA14$ could still be translated to generate a functional polypeptide.

Figure 4 Analysis of ATP synthesis rates in $\mu\Delta$ fibroblasts

Rates of ATP synthesis in $\mu\Delta$ fibroblasts are similar to those of control. Cells ($\mu\Delta$ and control fibroblasts) were digitonin-solubilized and ATP synthesis was measured as detailed in the Experimental section. No significant difference in the rates of ATP synthesis was detected in digitonin-permeabilized ($20 \mu\text{g/ml}$) cells (hatched bars), and the presence of oligomycin ($20 \mu\text{g/ml}$) strongly inhibited this activity (black bars). The experiments reflect independent repetitions of control 1 ($n=5$), control 2 ($n=4$) and $\mu\Delta$ ($n=11$).

Western-blot analyses demonstrated that not only is ATPase 6 synthesized from $\mu\Delta RNA14$ but also steady-state levels are comparable with the controls. This would suggest that the resultant translation product is not rapidly degraded. Moreover, had the ATPase 6 been translated with polylysine at the C-terminal as a consequence of mitoribosomal read-through, the translation product might be expected to migrate aberrantly during electrophoresis, whereas it paralleled that of the control samples. The pattern of *in vivo* mitochondrial translation products was consistent with an unaffected production of ATPase 6, and the only defect identified of the natural function of this protein was a subtle assembly fault of complex V. These results suggest that $\mu\Delta RNA14$ is not only recognized by the translation machinery, but a stable and apparently normal polypeptide is synthesized. The rapid de-adenylation of $\mu\Delta RNA14$ is translation-dependent and the length of the poly(A) tail before translation is 45–56 nucleotides. If the translating mitoribosome was capable of processing through the full length of this sequence, displacing putative poly(A) binding proteins, an additional 15 or so lysine residues would be expected to extend the C-terminus of ATPase 6. The absence of results supporting an additional 2–3 kDa to ATPase 6 suggests that the mitoribosome does not extend through the tail, but perhaps stalls as it encounters proteins bound to the poly(A) tail. As these proteins are displaced, RNases could access and digest the uncovered tail, allowing the mitoribosome to process to the truncated terminal where the mitoribosomal A site will become permanently free, mimicking a partially stalled complex. How, then, would the peptidyl tRNA molecule at the P site be hydrolysed and released, and the mitoribosome recycled?

The molecular mechanism of translation-elongation in mammalian mitochondria and *Escherichia coli* is believed to be similar [26], and there is some evidence that similarities are shared between translational-termination factors. Human mitochondrial release factor 1 (mtRF1) has been cloned and it appears to be more closely related to prokaryotic factors compared with the mitochondrial isoforms from lower eukaryotes. The putative tRNA mimicry domain is highly conserved and it has also retained the important GGQ motif [27], which is essential

for ribosome binding and release activity [28]. In addition, the human mitochondrial ribosome recycling/release factor was identified following a BLAST search of the human expressed sequence tag database with the *E. coli* RRF sequence [28]. How these translation-termination factors would operate on a stalled ribosome is unknown. In eubacteria, the mechanism for rescuing stalled ribosomes is well documented. Reports have identified an RNA molecule that is present in all eubacteria, known as SsrA or tmRNA, which can function both as a tRNA and an mRNA. The process initially involves the tRNA Ala-like domain, where tmRNA enters the empty A site of the stalled ribosomes adding the charged alanine residue to the polypeptide by transpeptidation. A consequent conformational change allows translation of an open reading frame internal to tmRNA to commence, thereby tagging the nascent polypeptide with ten additional amino acids [15]. Although this causes release of the mRNA from the ribosomes, the non-native C-terminal tag is now recognized by multiple *E. coli* proteases, resulting in rapid proteolysis [29]. It would be tempting to believe that with its many similarities to bacteria, the mitochondrion has retained such an elegant mechanism for resolving translation defects. Our Western-blot analysis results, however, indicate that there is no loss of stability of the translation product, as would be predicted from a tmRNA-like pathway, and that the migration of ATPase 6 is not consistent with a C-terminal extension of either polylysine or from tmRNA-like tagging.

Various conflicting results cloud the issue of the general mechanism of translation-termination and conclusive evidence of direct decoding of stop codons by RFs is still lacking [30]. Bacterial systems utilize two RFs for three stop codons; RF1 has a PAT motif recognizing UAA/UAG and RF2 an SPF motif recognizing UAA/UGA. In contrast, the eukaryotic cytosol has only eRF1 (eukaryotic peptide chain release factor 1) to respond to three stop codons; moreover, it has been demonstrated that it is the eRF that provides termination codon specificity and not the ribosome [31]. Human mitochondrial gene expression takes advantage of four different stop codons (UAA, AGA, AGG and UAG), but only mtRF1 has been identified to date, which shows little sequence similarity to eRF1. So how does the single mtRF1 recognize such diverse termination codons? One possibility is that the RF does not recognize these codons but actually distinguishes and associates with empty A sites. This will occur when the A site covers a termination codon that is not recognized by any tRNA, leading to ribosome stalling, or, as is seen with $\mu\Delta RNA14$, when the mitoribosome has travelled to the terminus of an mt-mRNA. So if mtRF1 does indeed recognize an empty A site, how does this recognition result in hydrolysis of the peptidyl tRNA at the P site, release of the nascent peptide and dissociation of the mitoribosome?

The important RF motif GGQ, essential for ribosome binding [27] and suggested to play a role in ribosome-dependent hydrolysis of peptidyl tRNAs [28,32], is retained in all class 1 RFs including mtRF1. Bacterial RF2, which also incorporates the GGQ motif, has been crystallized alone and in association with the ribosome, producing dramatically different structures. This led to the hypothesis that the initial interactions are codon-independent, with RF2 and the ribosome actually associating through GGQ, promoting a change in ribosome conformation [30]. Mitochondrial RF1 may combine various features from these systems. Perhaps, as with RF2, mtRF1 initially associates with the mitoribosome via a GGQ-mediated interaction at the A site, inducing a conformational change, which leads to ribosome-dependent hydrolysis of peptidyl tRNAs and peptide release. The mitochondrial RRF would then interact with the complex to disassemble the ribosome ready for re-initiation of translation. Although plausible, it is clear that much more work on the

functions of these release factors is required before this hypothesis can be rigorously challenged.

In conclusion, although the patient presented with symptoms consistent with mitochondrial disease and was investigated accordingly, at the time of the original publication [12] the patient had exceeded 15 months of age and it is believed that the individual is currently still alive. This, taken with the results presented here, suggests that, despite $\mu\Delta RNA14$ lacking a termination codon and being rapidly degraded, the mitochondrial machinery involved in processing, maturation, translation and ribosome release of the transcript can still recognize and process this substrate appropriately and that $\mu\Delta RNA14$ can function adequately to produce sufficient gene products for cell viability.

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