

Human mitochondrial ribosomes can switch their structural RNA composition

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The recent developments in cryo-EM have revolutionized our access to previously refractory structures. In particular, such studies of mammalian mitoribosomes have confirmed the absence of any 5S rRNA species and revealed the unexpected presence of a mitochondrially encoded tRNA (mt-tRNA) that usurps this position. Although the cryo-EM structures resolved the conundrum of whether mammalian mitoribosomes contain a 5S rRNA, they introduced a new dilemma: Why do human and porcine mitoribosomes integrate contrasting mt-tRNAs? Human mitoribosomes have been shown to integrate mt-tRNA^{Val} compared with the porcine use of mt-tRNA^{Phe}. We have explored this observation further. Our studies examine whether a range of mt-tRNAs are used by different mammals, or whether the mt-tRNA selection is strictly limited to only these two species of the 22 tRNAs encoded by the mitochondrial genome (mtDNA); whether there is tissue-specific variation within a single organism; and what happens to the human mitoribosome when levels of the mt-tRNA^{Val} are depleted. Our data demonstrate that only mt-tRNA^{Val} or mt-tRNA^{Phe} are found in the mitoribosomes of five different mammals, each mammal favors the same mt-tRNA in all tissue types, and strikingly, when steady-state levels of mt-tRNA^{Val} are reduced, human mitoribosome biogenesis displays an adaptive response by switching to the incorporation of mt-tRNA^{Phe} to generate translationally competent machinery.

mammalian mitochondria | ribosomes | rRNA | tRNA | mitochondrial protein synthesis

Mammalian mitoribosomes differ considerably from other ribosomes, primarily because of a reversed protein:RNA mass ratio, such that proteins predominate. Of the 80 known mitoribosomal proteins, 36 are mitospecific and lack bacterial orthologs, and those with orthologs often carry mitospecific extensions (1). Further idiosyncrasies have been revealed by recent subnanometer-resolution cryo-EM structures, which simultaneously resolved a long-debated question concerning mitoribosomal rRNA content (1, 2). Until recently, mammalian mitoribosomes were considered divergent by containing only one rRNA species per subunit, although the potential presence of the 5S species in the mitochondrial large subunit (mt-LSU) remained a contentious issue (3). Recent data have shown that there is indeed a third RNA moiety present in mammalian mitoribosomes. It is not, however, a 5S species, but a tRNA encoded by the mitochondrial genome (mtDNA) (1, 2, 4–6). Interestingly, which tRNA was selected differed between mammalian species, with mt-tRNA^{Val} predominating in human mt-LSU, in contrast to mt-tRNA^{Phe} being selected for incorporation into the porcine mt-LSU (Fig. 1 *A* and *B*).

Because mammalian mtDNA encodes 22 tRNAs (7), we sought to clarify whether incorporation into the mt-LSU is restricted to mt-tRNA^{Val} and mt-tRNA^{Phe}, or whether there is a more promiscuous use of mt-tRNAs in either a species- or tissue-specific manner. Furthermore, we investigated the consequence of reduced mt-tRNA^{Val} levels on human mitoribosomes.

Results and Discussion

Do Other Mammals Integrate Other Different Mitochondrially Encoded tRNAs into Their Mitoribosomes? Because the overall structure of tRNAs is generically considered to be similar, the level of discrimination displayed in the selection of mt-tRNA was perhaps surprising. To identify whether there was a process that integrated mitochondrially encoded tRNAs, other than mt-tRNA^{Val} or mt-tRNA^{Phe}, into the central protuberance of mammalian mitoribosomes, we investigated the mt-tRNA content of porcine, human, bovine, and rat mt-LSUs. Sucrose gradient separation of cell lysates facilitated analysis of the distribution of RNA and mt-LSU proteins within different fractions (Fig. S1 *A–C*). The predominant mt-tRNA comigrating with porcine and bovine mt-LSUs was mt-tRNA^{Phe} (Fig. 1 *C* and *E*), whereas mt-tRNA^{Val} was dominant in humans and rats (Fig. 1 *D* and *F*). The migration of other mt-tRNAs was determined, the positions of which are widely distributed across the encoding polycistronic transcript. None of these was substantially enriched among mt-LSU-specific fractions in any of the mammalian samples tested (Fig. 1 *C–F*). Our results indicate

Significance

Mammalian mitoribosomes are idiosyncratic. They reversed the typical 70% RNA:30% protein ratio to generate a protein-rich ribosome, which lacks the conventional 5S transcript as a third rRNA component. Recent subnanometer-resolution structures revealed a further unexpected eccentricity: integration of a mitochondrially encoded tRNA into the large-subunit structure. However, mitochondrially encoded tRNA (mt-tRNA) selection varies with mt-tRNA^{Val} integration into human mitoribosomes compared with porcine mitoribosomes favoring mt-tRNA^{Phe}. Our analysis of mitoribosomes from multiple tissues isolated from different mammals found that of 22 mitochondrially encoded tRNAs, only mt-tRNA^{Val} or mt-tRNA^{Phe} is incorporated. Further, there was no evidence of tissue-specific selectivity. Remarkably, human mitoribosomes can exhibit adaptive plasticity when mt-tRNA^{Val} levels are severely depleted by selectively integrating mt-tRNA^{Phe} instead, to generate translationally competent mitoribosomes.

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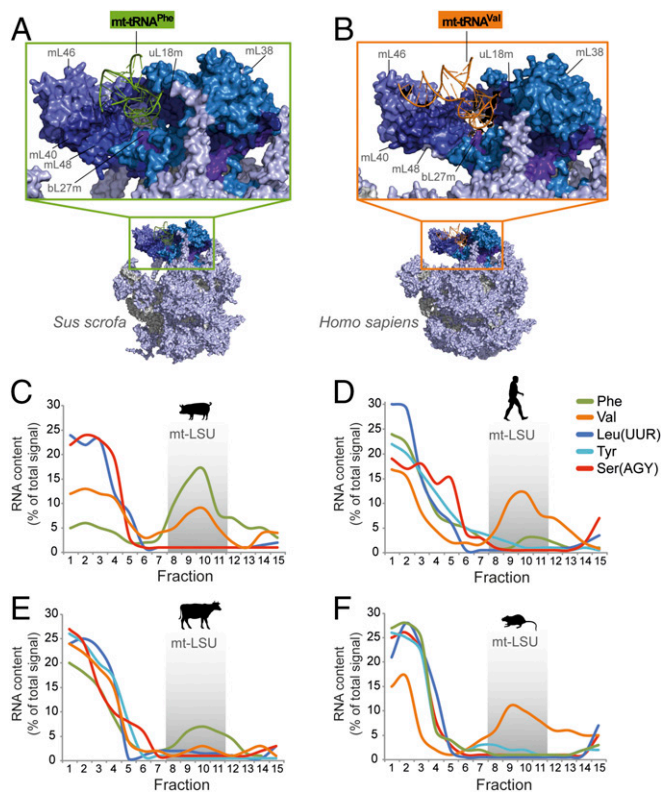


Fig. 1. Species- and tissue-specific variation in mt-tRNA incorporation into the mt-LSU was analyzed. Depicted are the overall structures of porcine (A) and human (B) mt-LSUs, detailing the region of the central protuberance containing mt-tRNA^{Phe} (porcine) or mt-tRNA^{Val} (human). The mitoribosomal proteins in direct contact with the mt-LSU-incorporated mt-tRNA are indicated in dark blue. Human (PDB: 3J9M); Porcine (PDB: 5AJ4). (C–F) To analyze and quantify species-specific mt-tRNA distributions, high-resolution Northern blots of sucrose gradients were performed. Analysis of porcine dermal fibroblasts (C), human osteosarcoma 143B cells (D), bovine cardiac tissue (E), and rat hepatic tissue (F) depicts the relative abundance of each mt-tRNA across fractions (ImageQuant software); represented as a percentage of the total signal.

that only mt-tRNA^{Val} or mt-tRNA^{Phe} are integrated into mammalian mitoribosomes.

Is There Tissue-Specific Variation in the Selection of mt-tRNA Integration? Having demonstrated species-specific incorporation of either mt-tRNA^{Val} or mt-tRNA^{Phe} into mammalian mitoribosomes, we addressed whether within a single organism there existed any tissue-specific variation. Mitoribosome sedimentation was performed for several mammalian tissues, and the ratio of free versus mt-LSU-bound mt-tRNA was quantified. Results indicated that mt-tRNA^{Phe} was the principal tRNA in porcine mt-LSU of cardiac, hepatic, skeletal muscle, and dermal epithelium (Fig. 2A). Notably, a fraction of mt-tRNA^{Val} was also detected in porcine mt-LSU across the tissues analyzed. In contrast, mt-tRNA^{Val} was consistently the dominant tRNA in the human, macaque, and rat mt-LSU across all tissues analyzed (Fig. 2B–D). These data suggest there is no tissue-specific incorporation of different mt-tRNAs into mitoribosomes.

Are the mt-tRNAs That Are Integrated as Structural Components Posttranscriptionally Modified? Mitochondrial tRNAs undergo extensive posttranscriptional nucleotide modification. The structural data identified that the mt-LSU-integrated mt-tRNAs were matured by the addition of CCA on the 3' terminus (6). We therefore sought to determine whether any differences in nucleotide modification status existed between these free and mt-LSU-bound

pools. No changes in m¹A9 modification were detected between the free versus integrated pools of human mt-tRNA^{Val} (Fig. S2). This is consistent with the mt-LSU structures (Fig. 1), where to integrate into the mt-LSU, mt-tRNAs adopt an L-shaped tertiary structure dependent on m¹A9 (8).

What Happens to Mitoribosome Biogenesis If Levels of mt-tRNA^{Val} Are Depleted? Despite mt-tRNAs making up only ~10% of the mitochondrial genome, more than 200 disease-causing mutations have been reported in these genes (9, 10). Among these pathogenic changes is a homoplasmic m.1624C > T mutation in the gene encoding mt-tRNA^{Val} (*MTTV*). The consequence of this particular mutation is a destabilization of the tRNA structure, which results in significantly reduced steady-state levels (11, 12). These depleted levels of mt-tRNA^{Val} were assumed to be the cause of compromised mitochondrial translation in patient tissue and cell lines (12). However, in light of the observation that mt-tRNA^{Val} is a component of the mitoribosome, we sought to determine the effect of reduced mt-tRNA^{Val} levels on mitoribosome formation.

Individual clonal lines were derived from a hybrid population that harbored the homoplasmic m.1624C > T mutation. To eliminate any conflicting contribution resulting from the aneuploid nature of 143B cells (13), five clones were analyzed. All shared a consistent biochemical and molecular phenotype, including decreased steady-state levels of mt-tRNA^{Val} compared with the parental 143B cell line (Fig. 3 depicts a representative clone). Experiments were designed to determine whether the protein synthesis defect was predominantly caused by decreased amounts of mitoribosome resulting from the reduced levels of mt-tRNA^{Val}, or whether both mitoribosome biogenesis and translation elongation were compromised by the available mt-tRNA^{Val} being distributed between integration into the mitoribosome and elongation, thus compromising both aspects of translation.

The redistribution of mt-tRNA^{Val} within sucrose gradients of the m.1624C > T hybrid lines was striking. The detectable majority

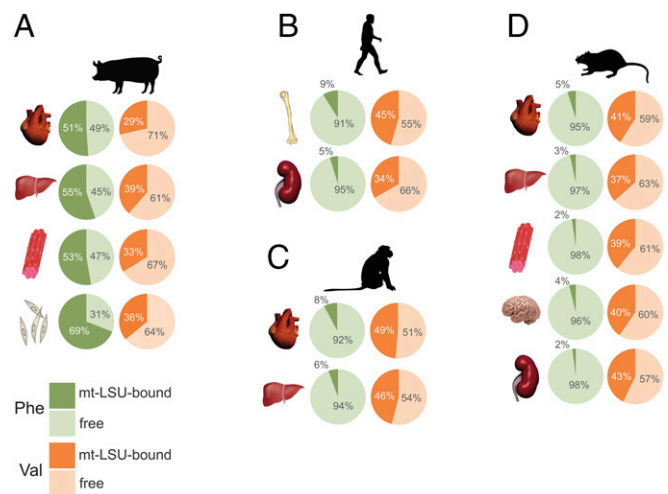


Fig. 2. Determination of tissue-specific variation in mt-tRNA incorporation into the mammalian mt-LSU. Selected tissues and cultured cells isolated from (A) pig (heart, liver, skeletal muscle, and dermal fibroblasts), (B) human (osteoblasts and embryonic kidney cells), (C) macaque (heart and liver), and (D) rat (heart, liver, skeletal muscle, brain, and kidney) were analyzed to determine any tissue-specific variation in mt-tRNA incorporation into the mt-LSU. Upon lysis, the samples were sedimented through sucrose gradients, followed by RNA extraction. RNA samples from fractions 1 and 2 (free pool) and fractions 10 and 11 (mt-LSU-associated) were subjected to Northern blotting. Abundance of mt-tRNA^{Phe} (green) and mt-tRNA^{Val} (orange) in free (pale green/orange), and mt-LSU-associated fractions (dark green/orange) is represented as a percentage of their combined signal.

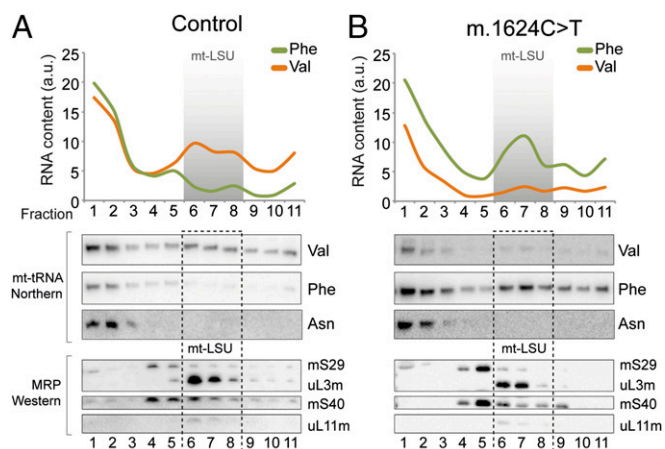


Fig. 3. The integration of mt-tRNA^{Val} or mt-tRNA^{Phe} into mitoribosomes of control cells or those harboring the *MTTV* mutation m.1624C > T was analyzed by sucrose gradient. Cell lysates from control 143B parental (A) and m.1624C > T (B) lines were separated through 10–30% (wt/vol) sucrose gradients and fractions collected (1 = top; 11 = bottom). Both RNA (Upper) and proteins (Lower) were extracted and analyzed the relative positions of mitoribosomal proteins (MRP) and mt-tRNA^{Val}, mt-tRNA^{Phe} with mt-tRNA^{Asn} as a control. The representative images are, in each case, the same membranes sequentially probed for different mt-tRNAs or MRPs.

was shifted to the free fractions compared with mt-LSU association in the 143B parental controls (Fig. 3, lanes 1–3; Fig. S3). This was consistent with the majority of the available mt-tRNA^{Val} being preferentially retained for use in elongation. Western blotting of the sucrose gradient fractions showed comparable levels and distribution of mitoribosomal proteins between the mutant and control lines. This indicated that the levels of mitoribosome were not directly proportional to the levels of mt-tRNA^{Val}, despite this element normally being required as a structural component.

To examine whether the mitoribosomes had formed in the absence of any mt-tRNA component, we probed the distribution of the remaining mt-tRNAs. All mt-tRNAs were analyzed and retained the same profile in control and m.1624C > T cells, with the majority present in the fractions corresponding to the free pool, available for aminoacylation and elongation. The notable exception was mt-tRNA^{Phe}, which, in contrast to 143B parental controls, now showed a significant proportion that partitioned with the mt-LSU (Fig. 3, lanes 6–8; Fig. S3). These data revealed that when the steady-state levels of mt-tRNA^{Val} are limiting, the human mitoribosome displays adaptive plasticity by integrating mt-tRNA^{Phe} into the mt-LSU.

We have shown previously that the molecular and biochemical defects in m.1624C > T-cell lines (11, 12) can be partially overcome by increasing levels of the cognate aminoacyl tRNA synthetase, VARS2, which stabilizes the mt-tRNA^{Val} (12). Therefore, to investigate whether suppression of the defect, observed with VARS2 overexpression, was caused by the expected redistribution of mt-tRNA^{Val} back into the mitoribosome, we analyzed independently generated lines with or without exogenous VARS2 induction (Fig. S4). Despite the increased availability of mt-tRNA^{Val}, biogenesis of the mt-LSU appeared to be sensitive to the structural abnormality in the mutant mt-tRNA^{Val}, as mt-tRNA^{Phe} was preferentially retained in the mt-LSU (Fig. S4). These data are consistent with the partial restoration of intraorganellar protein synthesis resulting from an increased pool of charged mt-tRNA^{Val} being available for elongation. High-resolution structures of mt-tRNAs, either in isolation or in complex with the mt-LSU, are currently lacking. It is, therefore, not possible to determine whether the preferential incorporation of mt-tRNA^{Phe} into the mt-LSU results from a better physicochemical fit than for the mutated

mt-tRNA^{Val}. However, translationally active mitoribosomes are generated, irrespective of which mt-tRNA is integrated (12).

Mammalian mitoribosomes have many features that make them distinct from other ribosomes, even compared with mitochondrial ribosomes from other organisms. Until recently, the apparent lack of a 5S rRNA was actively debated, particularly considering the level of conservation in its sequence, structure, and presence within ribosomes (14). Although the increased proportion of protein components in mammalian mitoribosomes is unusual, the substitution of the 5S for a tRNA is currently without precedent. The 5S rRNA conservation also extends to the location of its gene relative to other rRNA genes in the genome (15). Intriguingly, an analogous gene arrangement is found in most mammalian mitochondrial genomes, such that the two mt-tRNAs, which substitute for the 5S moiety in the mitoribosome, flank the two mt-tRNA genes (Fig. S5). This gene arrangement in mammalian mtDNA means that the polycistronic transcription unit encompassing both the 12S and 16S mt-rRNA, which is reportedly synthesized in a major excess over other units (16), would always include both mt-tRNA^{Phe} and mt-tRNA^{Val} (17). This infers stoichiometric availability of these RNA species for incorporation into mt-LSU. Our understanding of mitoribosome biogenesis is too rudimentary to identify when the mt-tRNA is incorporated, but on the basis of the positioning of the cleaved termini of the 16S and the mt-tRNA^{Val}, it seems unlikely that a polycistronic element is incorporated into the mt-LSU and subsequently cleaved. The context for CCA addition once integrated in the mt-LSU would be very different to all of the other mt-tRNAs, perhaps suggesting this occurs before incorporation, and therefore not when the mt-tRNA is part of a polycistronic unit. Because we know that certain mammals and, under certain circumstances, human mitoribosomes incorporate mt-tRNA^{Phe} in preference to mt-tRNA^{Val}, it seems unlikely that a ~2.5-knt RNA would be associated with the assembling mt-LSU before a cleavage event that would release the required mt-tRNA^{Phe}.

The mammalian mitoribosome has substituted an mtDNA-encoded tRNA for the 5S rRNA as a third structural RNA component. It has been well documented that mitochondrial RNase P is a protein-only complex that does not require RNA import into the mitochondrial matrix (18). Together, these observations support the absence of a functional requirement for RNA import into mammalian mitochondria.

In summary, the structural evidence for mt-tRNA incorporation, and the conserved positions of mammalian mtDNA genes, suggests an evolutionary significance of gene arrangement that would promote coordinated transcription of the RNA species destined to be structural components of the mt-LSU. The inclusion of two mt-tRNAs within this highly transcribed unit provides a degree of plasticity to the process of mitoribosome biogenesis, as seen by the differing use between organisms and the adaptive response observed under conditions of limiting mt-tRNA^{Val}. What controls the selection of a specific mt-tRNA, despite the relative similarities in structure, or whether other mt-tRNAs can be used when levels of both these species are limiting is yet to be determined.

Materials and Methods

Cell Culture. All cell lines used in this study were standard, commercially available cell lines except for the m.1624C > T transmittochondrial cybrid 143B lines, which used cytoplasts from patient cell lines that were derived from donated tissue samples. Access to samples that were excess to diagnostic requirements and were approved for research was covered by the license “Role of mitochondrial abnormalities in disease” (REC ref 2002/205) issued by Newcastle and North Tyneside Local Research Ethics Committee. Human 143B.206 Rho⁺ cells and cybrid derivatives were cultured [37 °C, humidified 5% (vol/vol) CO₂] in DMEM (Sigma) supplemented with 10% (vol/vol) FCS, 1× non-essential amino acids, and 2 mM L-glutamine. Where appropriate, Blastidicin^S (10 μg/mL) and Hygromycin^B (100 μg/mL) were added to select for successful integration and retention of the VARS2 gene. VARS2 induction was achieved by addition of 1 μg/mL tetracycline for 14 d.

Mitochondrial Transcript Analysis. Total RNA from the relevant tissues was extracted using TRIzol (ThermoFisher Scientific) and treated with Turbo DNase, following manufacturer's protocols. Northern blot analysis was essentially as previously performed (19), total RNA was resolved on 6% or 15% (wt/vol) UREA-PAGE (mt-tRNAs), subjected to wet transfer in 2× SSC (150 mM sodium chloride and 15 mM sodium citrate at pH 7.0) to a nylon membrane (MAGNEPROBE, 0.45 μ ; GE Healthcare or Genescreenplus, NEN DuPont). After UV-crosslinking (0.120 J), membranes were hybridized with radioactive probes, corresponding to mt-tRNAs, overnight at 65 °C in 7% (wt/vol) SDS and 0.25 M sodium phosphate buffer (pH 7.6), washed with 1× SSC three times for 20 min and then three times with 1× SSC containing 0.1% SDS (20 min; 65 °C). The membranes were exposed to a storage phosphor screen (GE Healthcare) visualized using a Typhoon phosphor-imaging system and quantified using ImageQuant software (Molecular Dynamics, GE Healthcare) or ImageJ (<https://imagej.nih.gov/ij>).

To analyze modifications, RNA was extracted using TRIzol reagent (ThermoFisher Scientific), following the manufacturer's instructions, from sucrose gradient fractions as indicated. The reverse transcription primer extension (RT-PEX) was as in ref. 20, the specific 5' ³²P-labeled primer (0.1 pmol) was incubated with 0.5–1 μ g of the RNA isolated from sucrose gradient fractions and subjected to reverse transcription reactions, as described by ref. 21. The reaction mixture was separated by 12.5% (vol/vol) PAGE containing 7 M urea, and dried gels were exposed to a storage phosphor screen and scanned as earlier. The following primers were used

in RT-PEX: mt-tRNA^{Val}: 5'-GTAAGTTGGGTGCTTTGTGTT, and mt-tRNA^{Phe}: 5'-TCAGTGATTGCTTTGAGGAGGT.

Analysis of Mitochondrial Ribosomal Protein Profile on Isokinetic Sucrose Density Gradients. Mitochondria were isolated from indicated tissues, as previously described (22). Cultured cells or mitochondria isolated from the tissues were lysed in buffer containing 50 mM Tris-HCl (pH 7.2), 1% Triton X-100, 10 mM Mg(OAc)₂, 100 mM NaCl EDTA-free complete protease inhibitor (Roche), and 0.08 U/mL RNasin (Promega). The lysates (0.7 and 0.1 mg of cell and mitochondrial lysates, respectively) were loaded on a linear sucrose gradient [2 mL 10–30% (vol/vol)] in 50 mM Tris-HCl (pH 7.2), 10 mM Mg(OAc)₂, 80 mM NH₄Cl, 0.1 M KCl, 1 mM PMSF, and centrifuged for 2 h 15 min at 100,000 g max at 4 °C (39,000 rpm; Beckman Coulter TLS-55 rotor). Fractions (15 × 100 μ L) were collected and 10 μ L aliquots were analyzed directly by Western blotting with the following antibodies; bl12m (PTG laboratories 14795–1-AP), uL3m (Abcam ab39268), mS29 (Abcam ab11928), mS40 (PTG laboratories 16139–1-AP), uL11m (CST D68F2).

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