CMLS, Cell. Mol. Life Sci. 60 (2003) 1356–1375
1420-682X/03/071356-20
DOI 10.1007/s00018-003-2343-1

CMLS Cellular and Molecular Life Sciences

Review

Human mitochondrial tRNAs in health and disease

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Received 3 December 2002; received after revision 14 January 2003; accepted 27 January 2003

Abstract. The human mitochondrial genome encodes 13 proteins, all subunits of the respiratory chain complexes and thus involved in energy metabolism. These genes are translated by 22 transfer RNAs (tRNAs), also encoded by the mitochondrial genome, which form the minimal set required for reading all codons. Human mitochondrial tRNAs gained interest with the rapid discovery of correlations between point mutations in their genes and various neuromuscular and neurodegenerative disorders. In this review, emerging fundamental knowledge on the structure/function relationships of these particular tRNAs and an overview of the large variety of mechanisms within translation, affected by mutations, are summarized. Also, initial results on wide-ranging molecular consequences of mutations outside the frame of mitochondrial translation are highlighted. While knowledge of mitochondrial tRNAs in both health and disease increases, deciphering the intricate network of events leading different genotypes to the variety of phenotypes requires further investigation using adapted model systems.

Key words. tRNA; mutation; aminoacylation; translation; structure; proteome; neurodegenerative disorders.

Introduction

Normal and pathological activities of human mitochondria are dependent both on the expression of the mitochondrial (mt) genome – a small circular DNA coding for 13 proteins, 22 transfer RNAs and 2 ribosomal RNAs (rRNAs) – and on the expression of numerous nuclear genes whose products are imported into mitochondria. These are estimated as 1000–2000 proteins and possibly several RNAs. The imported proteins include metabolic enzymes (mitochondria host numerous metabolic pathways, including the oxidative phosphorylation pathway, citric acid cycle, fatty acid degradation, heme biosynthesis and urea cycle [1]), and actors necessary for maintenance and expression of the mt genome. Replication and transcription exclusively depend on nuclear-encoded proteins. Translation is of dual origin, rendering this process of particular interest (fig. 1). Most partners of the mitochondrial protein synthesis machinery such as aminoacyl-tRNA synthetases, initiation, elongation and termination factors, ribosomal proteins, as well as enzymes involved in maturation and post-transcriptional modification of tRNAs, are nuclear encoded. Alternatively, the key molecules of this process, namely the set of 22 tRNAs and 2 major rRNAs, are of mitochondrial origin. The dual origin of the macromolecular partners in mitochondrial translation raises numerous unsolved questions concerning their characteristics, interactions, coordinated regulation, stoichiometry and activity. Mt tRNAs are central to these processes. The 13 mitochondrial translation products are all subunits of the respiratory chain complexes localized in the inner mitochondrial membrane, and are complemented by about 70 addi-

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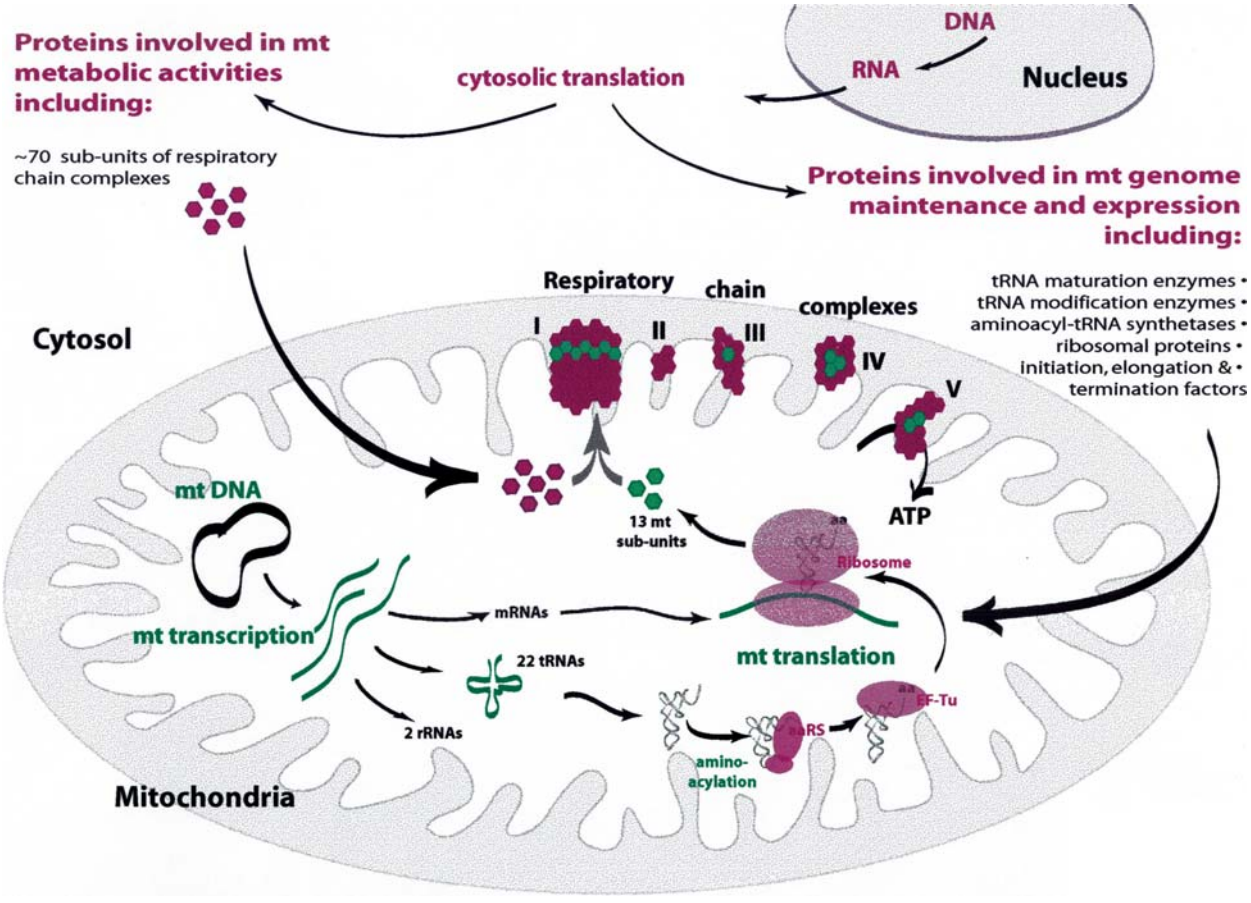

Figure 1. Dual origin of the human mitochondrial translation machinery. The mt genome codes for 22 tRNAs and 2 rRNAs (highlighted in green), while the nuclear genome encodes all other macromolecules involved in the mitochondrial translation machinery (in pink). Translation of the mitochondrial protein genes leads to 13 subunits (green hexagons) of the respiratory chain complexes. These are complemented by additional 70 subunits (pink hexagons) translated from nuclear genes and imported to mitochondria from the cytosol. The respiratory chain complexes form a physical and biochemical borderline between the mitochondrial matrix and the cytosol. All other activities of mitochondria, including numerous metabolic pathways as well as genome maintenance and expression, are coordinated by macromolecular partners (mainly proteins) encoded by the nuclear genome and imported.

tional subunits of nuclear origin to form active complexes for ATP synthesis (fig. 1). Thus, the activity of tRNAs as key effectors in translation is linked to metabolic activity at the physical and biochemical borderline between the mitochondrial matrix and the cytosol.

Mt genomes undergo high rates of mutations (10-fold higher than the nuclear genome) (e.g. [2, 3]). Numerous single nucleotide polymorphisms have been taken advantage of to determine population movements and reconstruction of human prehistory (e.g. [4, 5]). Such polymorphisms are apparently harmless to the affected gene and gene product. On the other hand, a large number of mutations correlate with a range of moderate to severe neuromuscular and neurodegenerative disorders [6–11]. Strikingly, more than 50% of the characterized pathology-related mutations concentrate within the tRNA genes, which represent only 10% of the mt genome. Understanding the molecular events underlying the various genotype/phenotype relationships in these mt tRNA disorders requires investigation of mt tRNAs both within and outside the frame of mitochondrial translation.

In this review, the increasing fundamental knowledge on human (and by extension on mammalian) mt tRNAs in mitochondrial translation, revealing original characteristics, is first summarized. Emphasis is given to tRNA biosynthesis, tRNA structural characteristics and tRNA interactions with different partners of the protein synthesis apparatus. In a second part, point mutations in mt tRNA genes and the complexity of their relationship to a variety of diseases are recalled. Further, a survey of the present knowledge on the impact of pathology-related mutations on tRNA structure/function relationships in mitochondrial translation will emphasize the diversity of molecular effects so far detected. Finally, recent discoveries on wide-ranging effects of tRNA mutations outside translation are presented. Despite the variety of approaches applied and the diversity of impact sites of mutations investigated, only the ‘emerging part of the ice-

berg' corresponding to the molecular cascade events underlying the pathologies is even starting to become unraveled. Recent developments of animal model systems allowing for investigation of mt DNA mutations offer new perspectives for elucidation of mt genome-linked diseases.

Human mitochondrial tRNAs in health

tRNAs are key actors in protein biosynthesis

tRNAs are fundamental actors in the conversion of the 4-letter nucleotide alphabet into the 20-letter protein language. Their structural and functional characteristics have been established soundly over half a century, and rules for recognition and interaction with macromolecular partners are well established [12, 13]. However, while the partners of the translational machinery are well defined in bacteria and cytosolic eukaryotic systems, far less is known about mitochondrial systems, and especially about human and mammalian mitochondrial systems. Particular structural features of mt tRNAs from various organisms have been reviewed [14, 15]. In what follows, major features of mammalian systems are recalled.

Role of tRNAs in human mitochondrial translation

Mammalian mitochondria require a complete translational apparatus for the synthesis of only 13 proteins. These proteins are all subunits of the respiratory chain complexes, and are involved in electron transfer and ATP synthesis. Seven proteins correspond to subunits of NADH-ubiquinone oxidoreductase (complex I; ND1–6), one is the cytochrome *b* apoprotein of ubiquinone cytochrome *c* oxidoreductase (complex III; cytb), three are subunits of cytochrome *c* oxidoreductase (complex IV; CO1–3) and two are subunits of ATP synthase (complex V; ATP6, ATP8). Only complex II, succinate dehydrogenase, consists exclusively of nuclear-encoded proteins. The 13 mitochondrial-encoded proteins are strongly hydrophobic (about 60% aliphatic and aromatic amino acids), a property which may account for maintenance of the corresponding genes within the mt genome, while all others have been transferred to the nuclear genome [1]. According to this imbalanced amino acid composition, the relative functional importance of the 22 tRNAs is different in mitochondrial translation. Thus, some aminoacyl-tRNAs (e.g. Ser-tRNA^{Ser}, Ile-tRNA^{Ile}, Thr-tRNA^{Thr} and Leu-tRNA^{Leu}) are required at much higher steady-state levels than others (e.g. Cys-tRNA^{Cys}) [15].

tRNA biosynthesis

Human mt DNA sequence and gene organization were determined more than 20 years ago [16–18]. The 22 tRNA genes lead to 18 tRNAs each specific for one amino acid, and 2 tRNAs specific for leucine and to 2 tRNAs specific for serine. tRNA$^{Leu(UUR)}$ and tRNA$^{Leu(CUN)}$ decode UUR and CUN leucine codons, respectively, and tRNA$^{Ser(AGY)}$ and tRNA$^{Ser(UCN)}$ decode corresponding serine codons. The tRNA genes are distributed all along the 16.6-kb DNA, and punctuate of the protein-encoding genes [17]. Whereas 14 of the 22 genes are encoded on the light DNA strand (C-rich strand), the 8 remaining genes are encoded on the heavy strand. Transcription of the mt genome leads to two large primary transcripts covering total information of either of the DNA strands, and a third, smaller transcript, containing tRNA^{Phe}, tRNA^{Val} and tRNA$^{Leu(UUR)}$ and the 2 ribosomal RNA genes. The gene for tRNA$^{Leu(UUR)}$ contains a transcriptional termination signal.

To convert the precursor transcripts into functional tRNA molecules, several processing steps are required, leading to the liberation of messenger RNAs (mRNAs), rRNAs and tRNAs (fig. 1). An RNA-containing enzyme (RNase P) is responsible for the generation of the mature 5′-end of tRNAs (reviewed e.g. in [19–21]). The removal of tRNA 3′ sequences is done by an endonuclease cleaving immediately 3′ to the last nucleotide of the tRNA gene [22]. Further, tRNA nucleotidyl transferase adds a 5′CCA3′ sequence, non-coded but necessary for the activity of tRNAs. A specific processing mechanism has been demonstrated for the two overlapping tRNA^{Tyr} and tRNA^{Cys} sequences within the precursor transcript [23]. In the larger frame of mammalian mt tRNAs, a specific post-transcriptional editing event has been reported once, in the case of marsupial tRNA^{Asp} anticodon sequences [24].

Structural characteristics

Structure is the basis for function. tRNAs have structural characteristics including a cloverleaf-like secondary structure and an 'L'-shaped three-dimensional (3D) structure [12]. The cloverleaf has well-defined characteristics including size of stems and loops and the presence of a number of conserved nucleotides at strategic positions. The 'L'-shaped 3D structure is based on an intricate, precise and conserved network of tertiary interactions. Mt tRNAs from different organisms present structural features which deviate more or less strongly from the classical ones [14]. While some show cloverleaf structures very close to the canonical situation, as is the case for *Saccharomyces cerevisiae* mt tRNAs, others are referred to as 'bizarre' tRNAs. The most striking examples concern *Caenorhabditis elegans* mt tRNAs where most are missing complete domains of the cloverleaf [25]. Human mt tRNAs fall into both of these categories, as will be detailed below. The exponential availability of fully sequenced mammalian mt genomes in genome databases has allowed for enlarged vertical comparisons of

large numbers of tRNA sequences. A study performed on 679 tRNA gene sequences from 31 fully sequenced mammalian mt genomes, classified into 22 families according to their amino acid specificity, allowed the prediction of 1, 2 and 3D characteristics of each family [26], thus leading to better-defined characteristics of human mt tRNAs.

Overall primary sequence properties

Due to the large difference in nucleotide composition of the two strands of the human mt genome, a subset of tRNAs can be considered as 'heavy tRNAs' (those transcribed from the light DNA strand), while the others are 'light tRNAs' (transcribed from the heavy DNA strand). The nucleotide composition of the two categories of tRNAs is summarized in table 1. Light tRNAs are deficient in G residues, and heavy tRNAs are deficient in C residues. In consequence, a higher number of CpA and UpA dinucleotides (nucleotide sequences very sensitive to degradation [27]) are found in light tRNAs, and the establishment of non-classical G•U pairs is more common in the heavy tRNAs [15]. Alignments of each of the 22 primary human mt tRNA sequences with the corresponding sequences in other mammalian genomes [26] revealed strong nucleotide conservation (e.g. tRNAs specific for methionine, leucine) as well as large variations (e.g. tRNAs specific for aspartic acid). Further, while all classical tRNAs share a set of strictly conserved or semi-conserved residues at precise positions, this is only partially the case for human and mammalian mt tRNAs (fig. 2A). Since most of these nucleotides are involved in 3D folding, they will be discussed below.

Table 1. Nucleotide composition of human mitochondrial tRNA genes.

tRNAs*	Average content per tRNA molecule					
	Total number of nucleotides†	A	C	G	U	Py-A
Light	71.3	25.7	16	10	19.6	12.8
Heavy	71.9	17.6	11.8	19.8	22.7	7.8

* Light tRNAs: Arg, Asp, Gly, His, Ile, Leu(CUN), Leu(UUR), Lys, Met, Phe, Ser(AGY), Thr, Trp, Val. Heavy tRNAs: Ala, Asn, Cys, Gln, Glu, Pro, Ser(UCN), Tyr.
† Total nucleotide content includes the 3′CCA end although not encoded by the mt genome.

Secondary and tertiary structures

Twenty out of 22 human mt tRNAs fold into cloverleaf secondary structures, however, with nonclassical dimensions for loop domains (fig. 2A, B). The two remaining tRNAs deviate more significantly from the cloverleaf structure. Indeed, $tRNA^{Ser(AGY)}$ has a shortened connector 1 and misses the complete D domain, and $tRNA^{Ser(UCN)}$ has an extended anticodon stem (6 bp instead of 5) (fig. 2B). Despite this apparent structural diversity, all these tRNAs have to be recognized by protein partners of the translational machinery, and to fit into the ribosomal active sites. When making a comparison with the available mammalian mt tRNA secondary structures, all three types of foldings are maintained and confirm the highly variable sizes of D and T loops [26]. Comparison of the human mt tRNAs with the available human cytosolic tRNAs (which are of classical type [28]) highlights the following differences. The size of D loops varies from 1 to 10 (1 refers to the tRNA completely missing the D domain, leading to a final connection between the anticodon and the acceptor stem of 4 nucleotides) with a mean of 5 nucleotides. This is smaller than the average 5–7-nucleotide-long D loops of cytosolic tRNAs. The size of T loops varies from 5 to 9 with a mean of 5 nucleotides, also making a striking contrast with the strictly conserved 7 nucleotides in classical tRNAs. The D and T stems also often vary strikingly in size. Interestingly, the so-called variable region containing 3–23 nucleotides in the case of classical tRNAs and 4–19 in human cytosolic tRNAs is restricted to 4–5 nucleotides in human mt tRNAs. Further secondary structure characteristics are the presence of weak GU pairs and of mismatches in helical domains. Light tRNAs are rich in mismatches (mean of 1.1 mismatch per tRNA) and poor in GU pairs (mean of 0.4 per tRNA); in contrast, heavy tRNAs are GU rich (3.9 pairs per tRNA), and poor in mismatches (0.1 per tRNA). This leads to a higher structural flexibility of all the tRNAs. Some of the mismatches are highly conserved in all mammalian mt tRNAs with a given amino acid specificity. For example, those mismatches located in the acceptor domain of $tRNA^{Met}$ are highly conserved (also at the level of the primary sequence).

The search for networks of tertiary interactions identical to those determined for classical tRNAs (based on the search for classically conserved nucleotides at strategic positions) was largely unsuccessful. Horizontal alignments of primary sequences of each tRNA gene family mainly highlighted the absence of a few strategic nucleotides (fig. 2A), especially nucleotides G18 and G19 in the D loop and nucleotides T54T55C56 of the T loop. Other elements (T8, A14, R15, A21, R26, R37, A58) are generally conserved within a specific tRNA family, and T33 is highly conserved among all mammalian tRNAs, except those for $tRNA^{Met}$. Only four families ($tRNA^{Leu(UUR)}$, $tRNA^{Leu(CUN)}$, $tRNA^{Gln}$ and $tRNA^{Asn}$) showed strong conservation of all nine potential classical tertiary interactions, including those between the D and T loops, suggesting a classical L fold (fig. 2C). For all other families, several of the classical tertiary interactions cannot occur (fig. 2C). Defining 3D interactions in these tRNAs remains a challenge, and only further experimentation will allow the alternate network of inter-

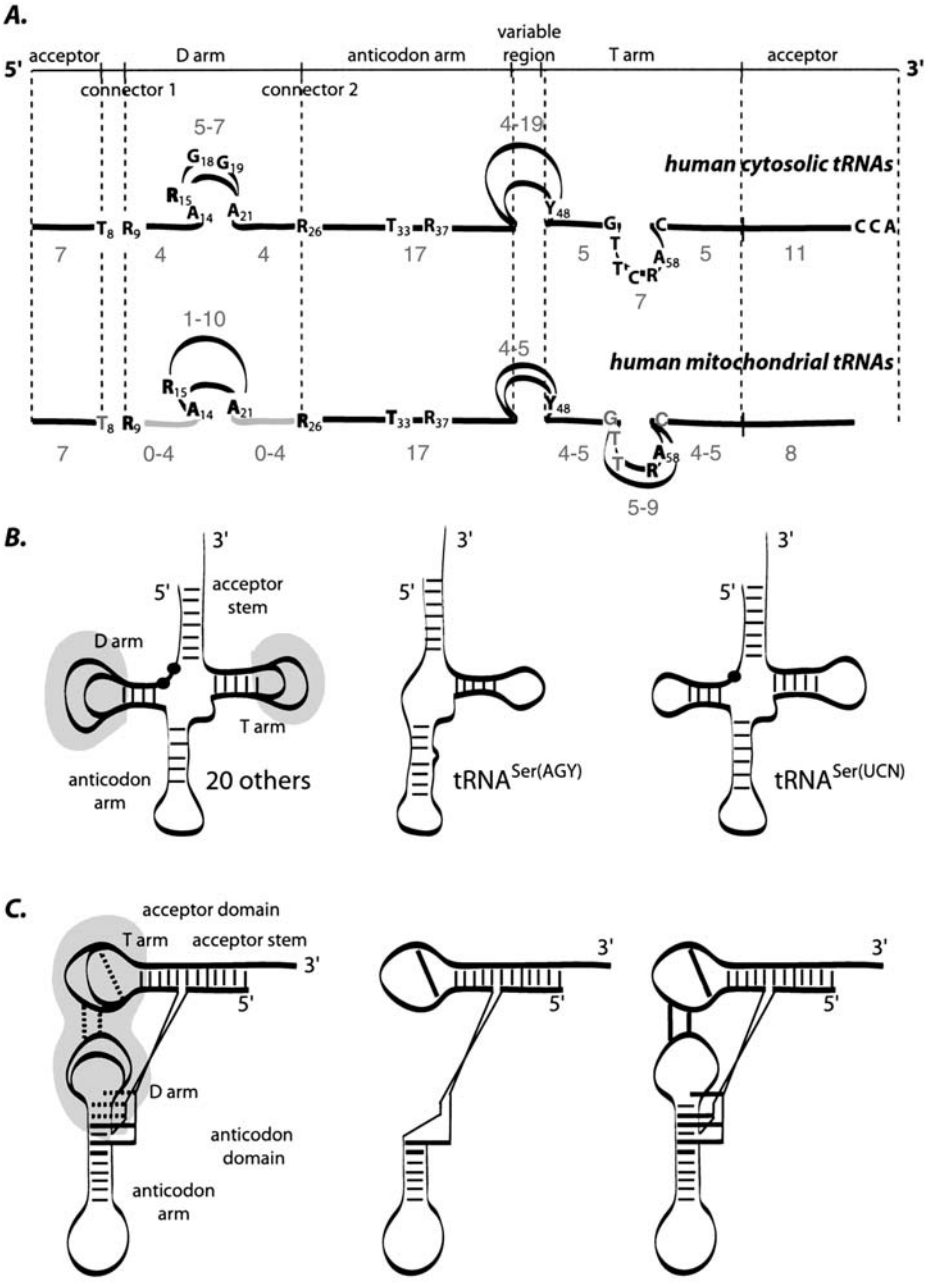

Figure 2. Structural properties of human mitochondrial tRNAs. (*A*) Linear organization of human mt tRNA genes as compared with canonical tRNA scaffolds in human cytosolic tRNAs. Names of tRNA structural domains are recalled in the upper part of the figure. Numbers correspond to sizes of the domains. Highly conserved and semiconserved residues (R and Y for purine and pyrimidine, respectively) are indicated. Size variations and degree of conservation of critical residues in mt tRNAs are emphasized by light-, middle- or dark-gray intensities (residues present in 50–70%, 70–90% or >90% of the mt tRNAs, respectively [26]). The CCA trinucleotide extremity is missing in the corresponding mitochondrial genes and is enzymatically incorporated posttranscriptionally. Thus, they are not indicated here. (*B*) Typical secondary structures of human mt tRNAs. Left, the general structure of 20 tRNAs, middle, the case of $tRNA^{Ser(AGY)}$ missing the complete D arm, and right, the case of $tRNA^{Ser(UCN)}$ which has a shortened connector 1 and an extended anticodon stem. (*C*) Schematic representations of the 3D structures. Long-distance interactions are displayed as bold lines, and nonstrictly conserved triple interactions are highlighted by dashed lines. Gray zones highlight domains where variations in the number and type of interactions differ from tRNA to tRNA.

actions underlying the 3D folding of each tRNA to be deciphered.

Experimental data on mammalian mt tRNAs remains scarce. Based on solution-mapping experiments with enzymatic and chemical probes, and/or on nuclear magnetic resonance (NMR) studies, models for 2D and 3D structures of bovine $tRNA^{Phe}$ [29], bovine $tRNA^{Ser(AGY)}$ [30] and bovine $tRNA^{Ser(UCN)}$ [31] have been proposed. Transient electric birefringence measurements showed an unusual angle between the branches of mt tRNAs [32, 33]. Structural studies on mt tRNAs prepared by in vitro transcription of cloned genes revealed that the human mt $tRNA^{Lys}$ folds into a functionally inactive bulged hairpin structure. Phylogenic comparisons as well as structural analyses of variant transcripts of this tRNA demonstrated that a single methyl group at position 1 of A9 (m1A9) can be sufficient to induce the cloverleaf folding of this unusual tRNA [34].

Posttranscriptional modifications

Like nuclear-encoded tRNAs, mt tRNAs are subjected to post-transcriptional modifications. Nucleoside modifications have been shown to be critical for both the structure and function of some classical tRNA species [35]. Presently, about 40 mammalian mt tRNAs have been directly sequenced, and 14 different modifications have been determined [28]. For human species, post-transcriptional modification contents and locations within 6 tRNAs have been identified (fig. 3). Most of these modifications are like those found in other tRNAs, but some are present at new positions. Thus, a methylation at an adenine at position 9 is only found in mt $tRNA^{Lys}$. Recently, two new modifications, namely taurinemethyl derivatives, specific to position U34 of the anticodon triplets of human mt $tRNA^{Lys}$ [36] and $tRNA^{Leu(UUR)}$ [37] have been described. In general, the extent of modification of mt tRNAs is lower than that of cytosolic tRNAs, suggesting that their role is of higher importance. As discussed above, this importance is illustrated by the role of m1A9 in $tRNA^{Lys}$, which hinders incorrect folding [34].

Interaction of mitochondrial tRNAs with partners

To fulfill their role in translation, tRNAs have to be recognized and to interact with a number of partners not only allowing their specific aminoacylation, but also their transfer to the ribosomal translation sites and interaction with the mRNA.

Mt tRNAs and aminoacyl-tRNA synthetases

During protein synthesis, tRNAs are recognized by specific aminoacyl-tRNA synthetases (aaRSs) which esterify the 3′ end of the tRNA with the corresponding amino acid. Several reviews have been devoted to the large body of knowledge on classical aminoacylation systems, namely those from prokaryotic organisms or from eukaryotic cytosol (e.g. [38–40]). Organellar and more specifically mammalian mitochondrial aminoacylation systems are reviewed in [41].

The 20 aaRSs are nuclear encoded and imported into human mitochondria. This leads to the question of how these enzymes can be distinguished from the set of cytosolic aaRSs. Most genes for the 20 human cytoplasmic aaRSs have been identified, and most of the corresponding enzymes have been studied. This is far from being the case for the mitochondrial synthetases. To our knowledge, 4 human mitochondrial aaRSs have been cloned, and the corresponding proteins overexpressed and characterized. These are LeuRS [42], LysRS [43], PheRS [44] and TrpRS [45]. In addition, sequence information and basic structural data on the corresponding genes are available also for GlyRS [46, 47], HisRS [48], IleRS [49] and bovine SerRS [50]. Progress in annotation of the human genome and identification of gene products will soon provide information for the missing aaRSs. Taking into account early observations, it might, however, be anticipated that no mitochondrial GlnRS will be found since no corresponding activity could be detected [51, 52]. For most of the known aaRSs, mitochondrial and cytoplasmic enzymes are encoded by distinct nuclear genes. Although the two enzymes share significant sequence similarities, these are lower than those observed between the mitochondrial and a prokaryotic aaRS (mitochondria are of prokaryotic origin). Noteworthy are the well-known GlyRS and LysRS cases, where mitochondrial and cytosolic enzymes are encoded by the same gene. For GlyRS, there are two initiation sites for translation, so that the two enzymes are only different at their N terminus, one enzyme having the mitochondrial-targeting signal, the other not [46, 47]. For LysRS, an alternative splicing pathway allows the insertion or not of the mitochondrial-targeting signal, leading to two mature aaRSs differing only by a few residues at their N terminus [43].

Mechanistic properties of mitochondrial aaRSs have not been fully investigated. For classical enzymes, their partition into two distinct classes has been established according to the structure of their catalytic domains. Two alternate and highly conserved structural modes of folding, responsible for the two possible mechanistic ways of amino acid activation and transfer, have been established [53]. Interestingly, the known mammalian mitochondrial aaRSs belong to the expected classes. tRNA recognition by aaRSs has been investigated by the search for possible cross-reactions with synthetases from other organisms. While not allowing for the establishment of a general rule, this approach did, however, delineate the trend for mitochondrial aaRSs to be able to aminoacylate heterologous tRNAs (*Escherichia coli,* mammalian cytosols), whereas mt tRNAs are not recognized by het-

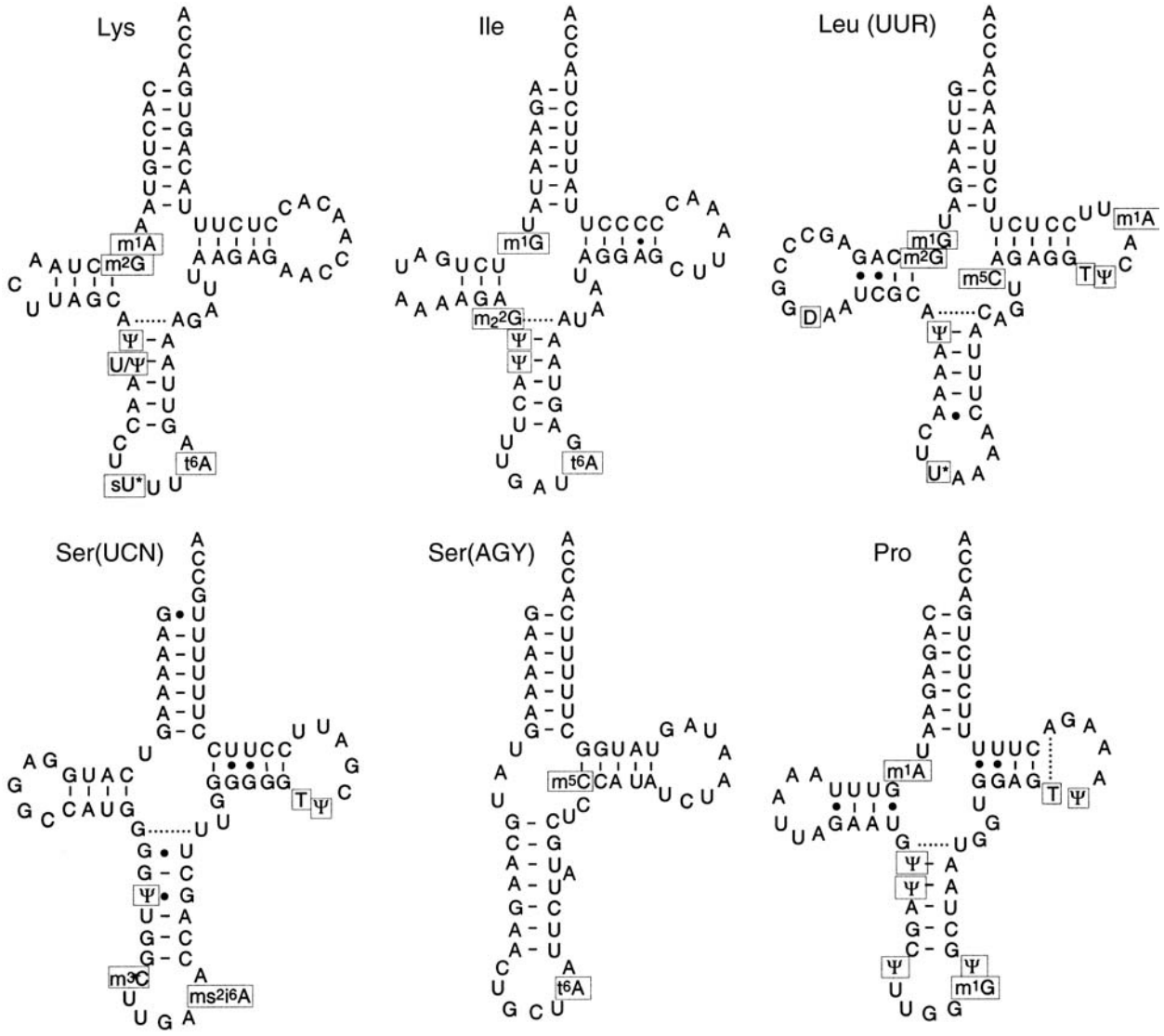

Figure 3. Human mitochondrial tRNAs and posttranscriptional modifications. tRNAs are displayed in their 2D cloverleaf representations. Posttranscriptional modifications are enclosed in squares. Abbreviations used are conventional, as in [28]. U* stands for taurinomethyl derivatives. References are indicated in the text and in table 2.

erologous synthetases (aaRS from *E. coli*) [54]. In addition, establishment of kinetic aminoacylation parameters highlighted that mitochondrial aaRSs are generally catalytically less efficient than cytosolic or prokaryotic homologues [42, 45]. Interestingly, $tRNA^{Ser(UCN)}$ and $tRNA^{Ser(AGY)}$ are aminoacylated by the same aaRS, although the structure of the two tRNAs is very different.

Specific recognition of a tRNA by its cognate aaRS is defined by precise rules and the presence of aminoacylation identity elements (e.g. [55]). A direct and systematic search for identity elements has been applied only to the case of bovine liver $tRNA^{Ser(AGY)}$. A dominant role, played by the T loop (especially nucleotide A58) and by nucleotides replacing the D loop, has been emphasized [56]. This is different from the identity elements found for the cytosolic serine system, where the presence of a large variable domain in the tRNA is necessary [50]. Information on a subset of elements involved in aminoacylation specificity is available in the case of marsupial $tRNA^{Asp}$, where it was found that the central nucleotide of the anticodon is a dominant identity element [57]. Indirect information on possible aminoacylation identity elements emerges from the analysis of primary molecular effects of mt tRNA pathogenic mutations correlated with neurodegenerative human diseases (see below). From a theoretical point of view, the nonconservation of the mitochondrial genetic code, as well as the particular structural features of mt tRNAs [26] and mitochondrial aaRS (e.g. [41]), suggest that aminoacylation rules may vary in mitochondria, compared with cytosol and other systems. A

significant example concerns alanylation, classically governed by a conserved identity base pair, G3-U70, in the amino acid-accepting stem of the tRNA, (e.g. [58–60]), a combination surprisingly absent in the corresponding human mt $tRNA^{Ala}$. The comparison of mammalian mt tRNA sequences highlighting conserved structural features and conservation of nucleotides at individual positions within the secondary structures has been explored as a theoretical source for identification of identity elements [26]. On the basis of the endosymbiotic origin of mitochondria, and of predicted conservation of major identity elements in evolution, it was found that most of the mt tRNAs contain the *E. coli* identity elements in large measure. The contribution of these elements to identity needs to be demonstrated experimentally, however.

Aminoacylated tRNAs interacting with initiation and/or elongation factors and with the ribosome

To initiate mitochondrial translation, the aminoacylated tRNA specific for methionine is formylated at the α-amine of the esterified amino acid by a tRNA transformylase, and further carried to the ribosome by the translation initiator factor IF2. Interestingly, in mammalian mitochondria, a single $tRNA^{Met}$ plays the role of initiator and elongator tRNA, raising the question how the use of this tRNA in these two steps of translation is regulated. Modulation of transformylase activity would probably meet the needs for formylated versus nonformylated Met-$tRNA^{Met}$ [61]. The bovine mitochondrial transformylase has been characterized and cloned [61] and the pattern of its interaction with the tRNA analyzed [62]. Elongation of translation is dependent on binding of the elongation factor EF-Tu to the aminoacylated elongator tRNAs. The bovine mitochondrial elongation factors EF-Tu and EF-Ts have been cloned [63], the affinity of EF-Tu for the tRNAs studied [64] and the crystal structure solved [65]. Elongation factor Tu recognizes the amino acid branch of classical tRNAs, composed of the amino acid acceptor stem and the T stem and loop [66]. Interestingly, in the nematode *C. elegans*, two elongation factors coexist, namely one specific for those mt tRNAs with a truncated T domain [67] and one specific for those missing the D arm [68]. In mammalian mitochondria a single EF-Tu binds all tRNAs, including $tRNA^{Ser(AGY)}$ lacking the D stem [68].

The mitochondrial ribosomes are formed by a small 28S subunit and a large 39S subunit [69]. They are characterized by a lower RNA content (33%) compared with bacterial ribosomes (65%), with a correspondingly larger number of protein subunits [70]. The small subunit consists of a 12S RNA and 29 proteins, while the large subunit consists of a 16S RNA and 48 proteins (mitochondrial ribosomal proteins, MRPs). The two rRNAs lack a number of well-defined secondary structure elements universally found in prokaryotic counterparts [71–73]. Numerous contributions based on 2D separation of proteins led to detection of new mitochondrial proteins ([72, 73] and references therein). About 50% (14/29 and 28/48) of these mitochondrial ribosomal proteins are homologous to those present in prokaryotes or in the cytosol of eukaryotes. However, the others are specific to mitochondrial ribosomes. Some of these proteins likely arose to fulfill additional mitochondria-specific functions. Thus, MRP-S29 was the first mitochondrial matrix protein reported to be involved in apoptosis [74] and MRP-S31 to be associated with type I diabetes [75].

While the mitochondrial ribosome has specific characteristics, all critical regions of its RNA and most of the proteins are conserved, suggesting a function analogous to that of bacterial or archaeal ribosomes. Not much is known about the interaction of the tRNAs in the ribosomal sites. However, the mt tRNA requires the presence of the homologous EF-Tu to become transferred to the A site. While mitochondrial ribosomes are expected to accept all mt tRNAs, despite their unusual structures, access to the ribosome of the bovine mt $tRNA^{Ser(AGY)}$ isoacceptor with a truncated D-domain is restricted [76].

Interaction of tRNAs with mRNAs

The human mitochondrial genetic code diverges slightly from the universal rules, with UGA codons for Trp instead of termination, AUA and AUU for Met instead of Ile and AGA and AGG for termination instead of Arg (fig. 4). Codon reading requires flexibility [77, 78]. This is particularly the case here since there are only 22 tRNAs available to read the 60 coding codons. Figure 4 summarizes the codons to be read by a given anticodon. In several boxes of the genetic code, each corresponding to a same amino acid, a single anticodon needs to bind 4 different codons. Indeed, 8 tRNAs with a U at wobble position 34 are able to read all 4 codons of the same group [Leu(CUN), Val, Ser(UCN), Pro, Thr, Ala, Arg, Gly]. Either codon reading is based on two base pairs instead of three, i.e. the wobble position would not interact with the 3rd nucleotide of the codon, or U is able to pair with any of the 4 nucleotides. However, 5 further tRNAs also have U at the wobble position, but read only those codons ending with A or G [Leu(UUR), Gln, Lys, Glu and Trp]. This rises the question why these U's are restricted from base-pairing with C or U. Likely they are post-transcriptionnally modified so as to prevent the flexibility required for such codon reading. In line with this interpretation, a modification at the wobble position has been demonstrated both for $tRNA^{Leu(UUR)}$ and for $tRNA^{Lys}$ [36, 37, 79, 80], and no modification of U34 was found for $tRNA^{Pro}$ [81] or $tRNA^{Ser(UCN)}$ [82].

A.

codon (5'-3')	(%)	amino acid	(nb)	anticodon (5'-3')	codon (5'-3')	(%)	amino acid	(nb)	anticodon (5'-3')	codon (5'-3')	(%)	amino acid	(nb)	anticodon (5'-3')	codon (5'-3')	(%)	amino acid	(nb)	anticodon (5'-3')
UUU	32.5	Phe	218	GAA	UCU	17.6	Ser	237	UGA $tRNA^{SerUCN}$	UAU	36.2	Tyr	146	GUA	UGU	26.6	Cys	22	GCA
UUC	67.5				UCC	42.7				UAC	63.8				UGC	73.4			
UUA	75.1	Leu	89	UAA $tRNA^{LeuUR}$	UCA	35.5				UAA		Ter			UGA	90.1	Trp	104	UCA
UUG	24.9				UCG	4.2				UAG					UGG	9.9			
CUU	12.2	Leu	553	UAG $tRNA^{LeuCUN}$	CCU	19.1	Pro	219	UGG	CAU	18.6	His	97	GUG	CGU	17.2	Arg	63	UCG
CUC	28.2				CCC	57.7				CAC	81.4				CGC	36.5			
CUA	50.2				CCA	19.8				CAA	87.8	Gln	90	UUG	CGA	41.4			
CUG	9.4				CCG	3.4				CAG	12.2				CGG	4.9			
AUU	39.4	Ile	321	GAU	ACU	15.4	Thr	357	UGU	AAU	23.4	Asn	164	GUU	AGU	26.7	Ser	56	GCU $tRNA^{SerAGY}$
AUC	60.6				ACC	46.1				AAC	76.6				AGC	73.3			
AUA	78.8	Met	207	CAU	ACA	35.6				AAA	88.4	Lys	95	UUU	AGA		Ter		
AUG	21.2				ACG	2.9				AAG	11.6				AGG				
GUU	21.1	Val	164	UAC	GCU	19.6	Ala	256	UGC	GAU	23.8	Asp	66	GUC	GGU	14.7	Gly	213	UCC
GUC	28.3				GCC	42.1				GAC	76.2				GGC	36.3			
GUA	38.2				GCA	33.9				GAA	68.3	Glu	88	UUC	GGA	32.8			
GUG	12.4				GCG	4.4				GAG	31.7				GGG	16.2			

B.

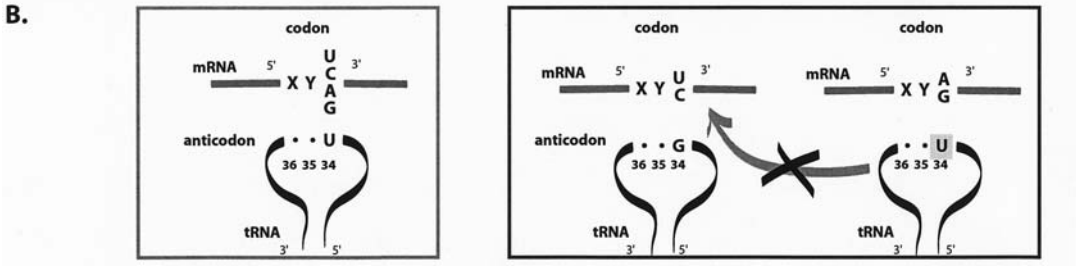

Figure 4. Human mitochondrial codon usage and codon/anticodon interactions. (*A*) Mitochondrial genetic code. Light and dark gray boxes highlight cases where a single anticodon needs to interact with four or two codons, respectively. In white boxes, only two codons are coding. Codon and anticodon triplets are given from 5′ to 3′. Percentage (%) corresponds to the relative use of each codon for one given amino acid. Numbers (nb) of amino acids are related to the need of each amino acid for translation of the 13 mitochondria encoded proteins. (*B*) Emphasis on cases where a U residue is at the wobble position (34) of the tRNA anticodon and has to interact either with 4 codons (light gray) or solely with 2 out of the 4 (dark gray boxes). The box with AUX codons (medium gray) is not considered since none of the corresponding anticodons has U34.

Mitochondrial tRNAs in disease

Correlation between tRNA gene mutations and neurodegenerative disorders

Ineffective mt DNA repair mechanisms and high oxidative stress are responsible for the high rate of mutations undergone by mt DNA (e.g. [2, 3]). Additionally, unlike yeast or plant mt genomes, the human mt genome displays intron-deprived, juxtaposed and sometimes overlapping genes, so that only 5% of the molecule is noncoding (transcription regulatory regions and replication origin). While some of these mutations are neutral (polymorphic mutations) and contribute to the diversity of human populations (e.g. [4, 5]), many mutations correlate with diseases (e.g. [6–11]). For simplification these are referred to as 'pathogenic mutations' in what follows.

In regard to tRNA gene mutations, 117 polymorphic [5], 87 pathogenic and 4 mutations with an ambiguous status have been reported over the last 13 years [83, 84]. All mutations are displayed in figure 5, on cloverleaf representations of the 22 mt tRNA genes (tDNA). Mutations can be located in any structural domain of the tRNA cloverleaf. Loops as well as stems are hit by substitutions. Noteworthy, however, is the almost total absence of substitutions in the anticodon triplet. The only known exceptions are the T12298C polymorphic mutation in $tRNA^{Leu(CUN)}$ and the G15990A pathogenic mutation in $tRNA^{Pro}$. Knowing the fundamental roles played by the anticodon (for codon reading and tRNA identity in a large number of systems), it is understandable that these residues are preserved, and most substitutions at the anticodon would be lethal. Several tRNA genes are hot spots for polymorphic mutations, while others are hot spots for pathogenic mutations. Polymorphic mutations are most found in the genes specific for $tRNA^{Thr}$ (16 mutations), $tRNA^{Asp}$ and $tRNA^{Arg}$ (8), $tRNA^{Cys}$, $tRNA^{Gln}$ and $tRNA^{His}$ (7). Pathogenic mutations affect predominantly genes for $tRNA^{Leu(UUR)}$ (20 mutations), $tRNA^{Lys}$ (10) and $tRNA^{Ile}$ (10). Up to now, 21 of the 22 tRNA genes are hit by disease-related mutations, the gene for $tRNA^{Arg}$ being so far the only exception.

The phenotypes related to pathogenic mutations are numerous, ranking from mild (e.g. exercise intolerance,

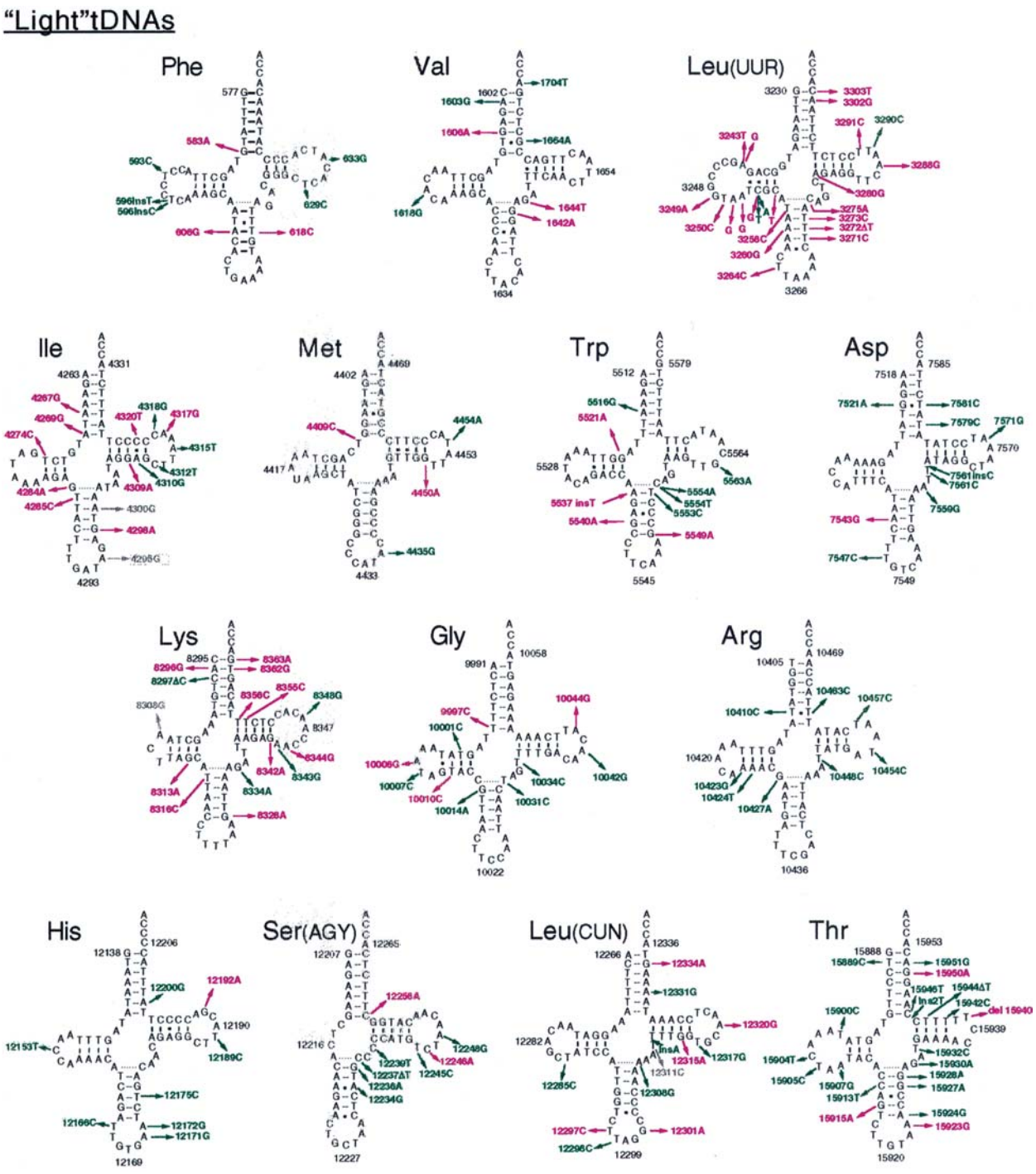

Figure 5. Pathogenic and polymorphic mutations in human mitochondrial tRNA genes. tRNA genes are displayed in a 2D cloverleaf representation and sorted according to their location in the mt genome. 'Light' and 'heavy' tDNAs correspond respectively to sequences on the G-poor or G-rich mt DNA strands. Numbering is according to the standard sequence [16]. Pathogenic mutations are indicated in pink, and polymorphic mutations are displayed in green. Mutations with an ambiguous status are indicated in black. Mutations were retrieved from updated databases [5, 83] (http://www.mitomap.org/ and http://www.genpat.uu.se/mtDB/). Ins and Δ stand, respectively, for a nucleotide insertion or deletion.

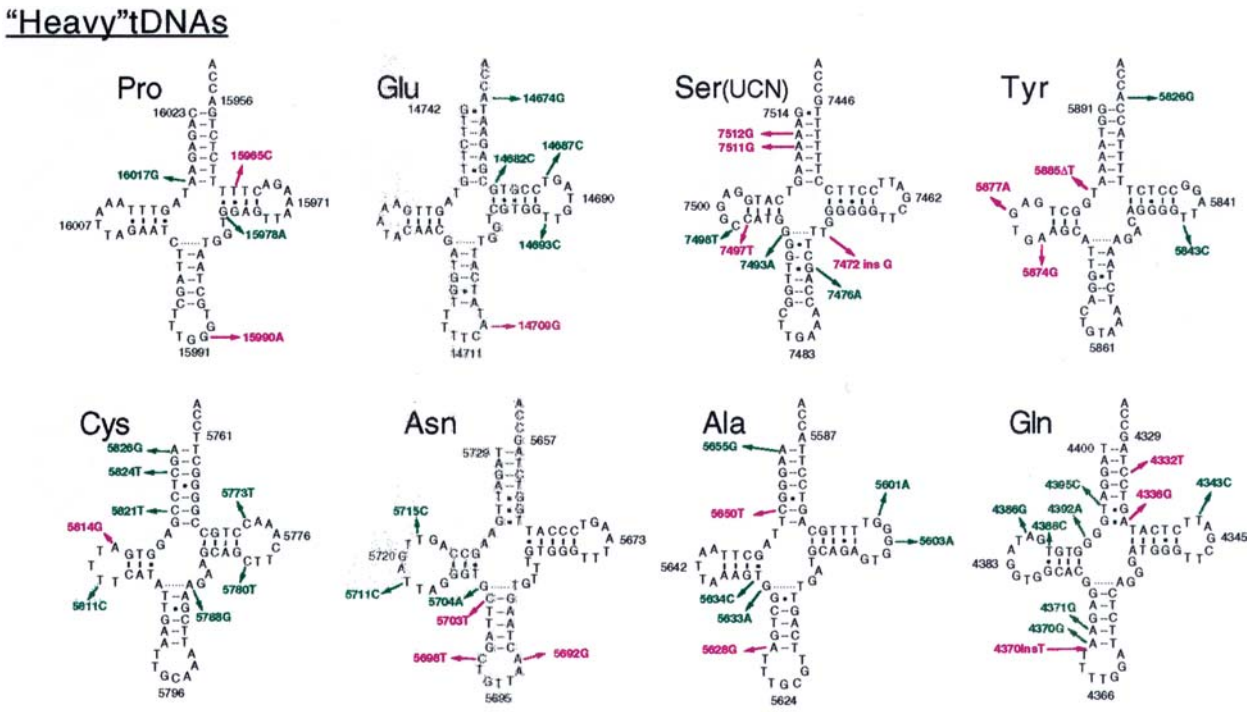

Figure 5 (continued).

limb weakness) to severe (even lethal), including muscular and/or neuronal disorders (e.g. myopathies, encephalomyopathies, cardiopathies, diabetes, ophtalmoplegia, deafness). The most prevalent mutations correspond to A3243G in $tRNA^{Leu(UUR)}$, correlated with MELAS (mitochondrial epilepsy with lactic acidosis and stroke-like episodes) and A8344G in $tRNA^{Lys}$, correlated with MERRF (myoclonic epilepsy with ragged red fibers) syndromes.

Complexity in genotype/phenotype relationships

The relationships between genotypes and phenotypes are complex (e.g. [7–9, 85]). Indeed, the same mutation can lead to different pathologies, while the same pathology can be correlated with different mutations. Some of these observations might be explained by mitochondria-specific features such as mitotic segregation of maternally inherited mitochondria (random tissue distribution of mutated mt DNA) and heteroplasmy (wild-type and mutated mt DNA coexist in the same mitochondrion or in the same cell). In addition, the disease phenotype is expressed only above a given relative proportion of mutated DNA. Despite this initial complexity, it is of importance to decipher the biochemical and molecular mechanisms linking any mutation in a tRNA gene to the corresponding pathological status of the affected cell. This will in turn allow the design of straightforward therapeutic approaches.

Initial biochemical features of tRNA-related pathologies concern energetic deficiencies. These are characterized by lower cellular respiration (oxygen consumption), lower activity of the respiratory chain complexes (often deficiency in complex I and complex IV activities) and lower ATP synthesis. Due to the central role of tRNAs in translation, leading to the synthesis of 13 mitochondrial subunits of the respiratory chain complexes, direct defects in mitochondrial translation were sought. A strongly decreased rate of mitochondrial protein synthesis was indeed shown for several individual mutations in different cellular backgrounds (e.g. [86–92]). The decreased respiratory chain activity is generally considered as linked to the lower abundance of mitochondrial encoded subunits or to the presence of nonfunctional subunits [7–9, 85]. However, exceptions where the rate of mitochondrial translation was unaffected (or only very mildly affected) by a tRNA gene mutation were also reported, suggesting that alternative (or additive) mechanisms associate a tRNA gene mutation with a phenotype [93, 94]. Numerous studies have focused on deciphering the initial molecular impact of mutations on the tRNA structure/ function relationship in translation. As summarized below, according to the mutation studied, different steps of the tRNA life cycle were found affected, demonstrating that at a molecular level, the genotype/phenotype relationship is also complex. Further studies allow wide-range secondary effects of the mutations outside of mitochondrial translation to be deciphered.

tRNAs malfunction in the context of mitochondrial translation

Experimentation on molecular impact of mutations during the tRNA life cycle

Investigation of molecular effects of mutations in mt tRNA genes is a difficult task principally due to restricted access to material and to the heteroplasmic nature of mitochondrial DNA. A first breakthrough came from the creation of couples of homoplasmic cybrid (cytoplasmic hybrids) cell lines, i.e. cells with the same nuclear background and with mitochondria distinguished only by the absence or presence of a specific mutation in a given tRNA gene [95]. As an alternative, some studies have been performed on patient biopsies or primary cell cultures. Finally, wild-type and mutated tRNAs have been prepared by cloning the corresponding synthetic genes and in vitro transcription. These different approaches have been described previously and will not be further discussed here (e.g. reviewed in [15, 96]).

Table 2 summarizes the various levels at which tRNA defects have been observed as a result of pathogenic mutations (and includes all references). Only about 15 of the 87 mutations have so far been investigated for one or more aspects of mitochondrial translation. Thus only 6 tRNAs have been considered, reflecting the technical difficulties discussed above. The best-studied mutations are those related to MELAS (A3243G) and MERRF (A8344G) syndromes. Mutations in the gene for $tRNA^{Leu(UUR)}$ were found to affect termination of transcription in generating a 19S RNA. A series of mutations in $tRNA^{Leu(UUR)}$ and in $tRNA^{Ser(UCN)}$ affect maturation of the primary transcript in interfering with RNaseP cleavage or 3′-tRNase cleavage, respectively. Further, some mutations interfere with post-transcriptional modification of specific nucleotides in the tRNAs; others do not. While no drastic structural changes have been observed in mutated tRNAs, the flexibility of mutated tRNAs is either restricted or increased, hindering their optimal interac-tion with molecular partners for functional means. It was recently reported in the case of MELAS mutation A3243G that the new nucleotide enables duplex formation between two tRNA molecules by intermolecular base-pairing between the tRNA D loops. The sole feature constant to all investigated mutations is their negative effect on tRNA stability. Indeed, all mutated tRNAs studied are degraded faster than the corresponding wild-type tRNAs.

In regard to tRNA function, the effects of mutations on the aminoacylation properties of tRNAs have been the most thoroughly investigated. A variety of surprising results have been obtained, leading sometimes to controversies. For example, while some mutations do affect aminoacylation, others do not. Typically, mutation 8344 does or does not affect aminoacylation depending on the nuclear background of the investigated cell, or according to the approach used for in vivo measurements. Determination of kinetic parameters of aminoacylation in the presence of purified enzyme showed that the mutation affects neither the rate of aminoacylation nor the affinity of the enzyme for its tRNA. The systematic comparison of the in vitro aminoacylation capacities of a series of $tRNA^{Ile}$-derived mutants revealed a large gradation in effects. In addition to the fact that mutations affect aminoacylation either weakly or severely, several mutants were shown to be competitive inhibitors of isoleucylation of wild-type $tRNA^{Ile}$. This is relevant to the heteroplasmy in patient cells, and highlights the possible negative effect of a mutation not only on the tRNA molecule carrying the mutation, but also on the 'healthy' corresponding tRNA. Interestingly, also, one mutant showed an increased aminoacylation efficiency relative to wild-type $tRNA^{Ile}$, due to increased global conformational stability.

Present understanding of the effects of mutations on aminoacylation suggests features idiosyncratic to each mutation/aminoacylation system, depending on the biological environment. As suggested by others [93, 97], aminoacylation may depend on additional factors (e.g. chaperones or cofactors, ...) as was shown to be the case for a subset of 'classical' tRNAs (e.g. [98]). Despite the variety of effects observed, searching for primary effects of mutations in human mt tRNAs at the level of the aminoacylation process remains of major importance in the frame of therapeutic approaches. Indeed, aaRSs, which are nuclear-encoded proteins, appear to be ideal tools at least at two levels. Transformation of affected cells with the corresponding genes would allow for higher steady-state levels of the synthetase in mitochondria, which could increase aminoacylation of the defective tRNA, as was shown in *E. coli* [99]. Alternatively, aaRSs may be used as shuttles for import of wild-type tRNA, which may compensate for the deleterious mutated molecules. Promising experiments along these lines have already been reported [100, 101].

In a further functional event, the aminoacyl-tRNA is carried towards the ribosome via interaction with translation elongation factor EF-Tu. Only one study of the possible effect of a mutation on the interaction with this factor has so far been performed. It was concluded that EF-Tu protects the mutated aminoacyl-tRNA about twofold less efficiently than it protects the wild-type aminoacylated tRNA from degradation, suggesting that the mutation interferes with optimal interaction with EF-Tu [92]. In a final step of protein synthesis, mutated tRNAs may become unable to bind mRNA. This effect has been reported for $tRNA^{Lys}$ with mutation A8344G. This mutation, located in the T arm of the tRNA, leads to complete absence of a post-transcriptional modification in the anticodon triplet at position 34 in HeLa cells [92]. Absence of this specific modification hinders anticodon/codon binding. Inter-

Table 2. Molecular effects of point mutation in mitochondrial tRNA biogenesis and functions.

Step in tRNA life Mutation	tRNA	Experimental system	Result	References
Transcription				
A3243G	Leu(UUR)	mitochondrial extract from human KB cell cultures	impaired termination	[133]
A3243G	Leu(UUR)	cybrid/osteosarcoma	impaired termination	[93, 134]
A3243G	Leu(UUR)	cybrid/osteosarcoma	no effect on mTERF binding	[91]
A3302G	Leu(UUR)	muscle, fibroblasts	impaired termination	[135]
A8344G	Lys	cybrid/osteosarcoma	no effect on transcription	[136]
A8344G	Lys	cloned myoblasts	no effect on transcription	[137]
T8356C	Lys	cybrid/osteosarcoma	no effect on transcription	[136]
Maturation				
A3243G	Leu(UUR)	SV40 transformed B cells	impaired RNAse P processing	[138]
C3256T	Leu(UUR)	in vitro transcript	impaired RNAse P processing	[20]
A3260T	Leu(UUR)	in vitro transcript	impaired RNAse P processing	[20]
T3271C	Leu(UUR)	in vitro transcript	impaired RNAse P processing	[20]
T7445C	Ser(UCN)	in vitro transcript	defect in 3′ end processing	[139]
Posttranscriptional modification				
A3243G	Leu(UUR)	cybrid/osteosarcoma	decrease in methylation	[80]
A3243G	Leu(UUR)	cybrid/HeLa	absence of modification of U34	[37]
T3271C	Leu(UUR)	cybrid/HeLa	absence of modification of U34	[37]
A4269G	Ile	cybrid/HeLa	no change	[140]
7472insC	Ser(UCN)	cybrid/osteosarcoma	no change	[82]
A8344G	Lys	cybrid/osteosarcoma	no change	[80]
A8344G	Lys	cybrid/Hela	absence of modification of U34	[36]
G15990A	Pro	in vitro transcript	decrease in methylation	[81]
Structure				
A3243G	Leu(UUR)	in vitro transcript	formation of duplexes	[141]
T4285C	Ile	in vitro transcript	global structural weakness	[142, 143]
G4298A	Ile	in vitro transcript	global structural weakness	[142, 143]
A4317G	Ile	in vitro transcript	global structural weakness	[142, 143]
C4320T	Ile	in vitro transcript	structural stabilization	[142, 143]
G5703A	Asn	cybrid/fibroblast	conformational alteration	[90]
7472insC	Ser(UCN)	cybrid/osteosarcoma	no effect	[82]
Stability				
A3243G	Leu(UUR)	cybrid/osteosarcome	decreased	[91]
A3243G	Leu(UUR)	cybrid/HeLa	decreased	[37]
T3271C	Leu(UUR)	cybrid/HeLa	decreased	[37]
A4269G	Ile	cybrid/HeLa	decreased	[140]
A8344G	Lys	cybrid/osteosarcoma	decreased	[88]
A8344G	Lys	in vitro transcript	decreased	[36]
Aminoacylation				
A3243G	Leu(UUR)	cybrid/osteosarcoma	decreased	[91 , 93]
A3243G	Leu(UUR)	biopsy	decreased	[57]
A4269G	Ile	in vitro transcript	decreased	[144]
T4285C	Ile	in vitro transcript	decreased	[142, 143]
G4298A	Ile	in vitro transcript	impaired aminoacylation, competitive inhibition of wt tRNA aminoacylation	[142, 143]
A4317G	Ile	in vitro transcript	decreased	[143, 145]
C4320T	Ile	in vitro transcript	increased	[142, 143]
A8344G	Lys	cybrid/osteosarcoma	decrease	[88]
A8344G	Lys	biopsy	no effect	[57]
A8344G	Lys	tRNA from cybrid/HeLa	no effect	[92]
Binding to elongation factor EF-Tu				
A8344G	Lys	tRNA from cybrid/HeLa	decreased protection by EF-Tu against degradation	[92]
Codon reading				
A8344G	Lys	cybrid/HeLa	impaired codon reading	[92]
G15990A	Pro	biopsy	conversion to serine anticodon	[144]

estingly, in lung carcinoma cybrids, a suppressor $tRNA^{Leu(CUN)}$ with a mutation at residue 12,300 (position 36 in the anticodon loop) compensates for deleterious effects of mutation A3243G in $tRNA^{Leu(UUR)}$ [102]. It has been proposed that the compensatory mutation allows for decoding UUR leucine codons while preventing misreading of UUC and UUU phenylalnine codons [78].

The individual effects reported for different mutations and/or different tRNAs could accumulate for a given mutation to lead to deleterious mitochondrial protein synthesis. This is the case when e.g. decreased stability and decreased aminoacylation efficiency lead to lowered steady-state levels of aminoacyl-tRNA available to ribosomes. This effect slows down ribosomal progression along the mRNA and leads either to polysomes of lower size [103] or to frameshifting events [104]. Alternatively, each individual effect on tRNA biogenesis or precise function may be sufficient to affect the whole translational machinery and lead to the same final effect, namely decreased rate of synthesis of respiratory chain subunits.

Theoretical considerations of pathogenic versus polymorphic mutations

The complexity of the genotype/phenotype relationships, diversity of molecular impacts of different mutations at the tRNA structure/function level and discovery of new mutations all call for a search for unifying features. Along this line, a comparison of the basic features (at the level of primary and secondary tRNA structure) of 68 pathogenic mutations and 64 polymorphic neutral mutations has been performed [105]. Both types of mutations appear to be distributed randomly. Most pathogenic mutations affect highly conserved nucleotides, whereas most polymorphic mutations affect rather nonconserved nucleotides, with some exceptions. The degree of conservation of a nucleotide thus cannot be taken as a guarantee that the mutation will be pathology related. Most mutations are transitions (conversion of pyrimidine into pyrimidine and of purine into purine), and those which affect stem regions, convert canonical Watson-Crick base pairs into mismatches, mostly C•A or G•U. Thus, despite the accumulation of information about the positions of a large number of mutations within mt tRNAs, it is not possible to identify simple basic features allowing the prediction of pathogenicity of new mutations. Therefore, systematic molecular investigations of pathogenic mutants need to be extended to a large number of cases to find unifying features at other levels.

Effects of mutations outside mitochondrial translation

Due to the link between mitochondrial- and nuclear-encoded proteins involved in the formation of the respiratory chain complexes, deleterious effects of a point mutation in a mt tRNA gene could further spread to the cytosol and its components and contribute to disease status. Wide-ranging consequences of a mt tRNA gene mutation have thus been studied.

Biochemical perturbations outside mitochondria

Biochemical consequences of mutations are numerous, and lowered mitochondrial membrane potential [106], less efficient calcium uptake by mitochondria and elevation of cytosolic steady-state calcium levels [107, 108] have been reported. The reverse communication from mitochondria to nucleus uses calcium-mediated pathways activating calcium/calmodulin-dependent kinase, which would phosphorylate the transcription factor CREB (c-AMP-responsive element-binding protein). The phosphorylated CREB would mediate a proliferation defect in mutation-carrying cells by an increase in transcriptional activity of specific tumor suppressor genes [109]. Mutated cells not only proliferate much more slowly than healthy cells, but also undergo higher levels of apoptosis. Apoptotic events, including decrease in membrane potential [110], DNA fragmentation caused by the accumulation of free radicals [111] and increased expression of apoptosis-associated proteins (e.g. Fas, p75 and caspase-3), have been reported in muscle biopsies of patients with mt tRNA gene mutations [112]. In the case of these mutations an imbalance of the relative abundance of nuclear-encoded respiratory chain subunits compared with mt DNA-encoded subunits occurs, which may favor cytochrome *c* inactivation and release. In consequence, cytochrome *c*, together with the respiratory chain dysfunction, could activate apoptotic pathways which in turn would lead to a decreased rate of mitochondrial translation and decreased import of nuclear-encoded mitochondrial protein precursors [112]. In addition to this 'classical' apoptotic pathway, unique apoptosis-related changes, which differ from the caspase-3-dependent mechanism, have been reported in muscle biopsies of a MELAS patient, while superoxides were overproduced and cytochrome *c* was released [113]. However, apoptosis can be suspended in muscle of MERRF and MELAS patients [114]. The authors describe the induction of cytochrome *c* release and the activation of caspase-3. However, only mild peripheral chromatin condensation, no DNA fragmentation and an increased expression of the human inhibitor of apoptosis protein were reported as compensatory mechanisms.

Perturbations in the mitochondrial proteome

To visualize the wide-ranging effects of a point mutation in the context of mitochondrial disorders, a global approach using comparative proteomics has been developed. This method allows both quantitative and qualitative variations of several hundred proteins to be investigated at once [115, 116]. The first comparative

proteomics study carried out on mitochondria from sibling human cybrid cell lines, differing only by a single point mutation in their $tRNA^{Lys}$ gene (A8344G associated with MERRF syndrome) or in their $tRNA^{Leu(UUR)}$ gene (A3243G associated with MELAS), has proven the feasibility of such an approach [117]. Two-dimensional gel electrophoresis (isoelectric focusing / SDS-PAGE) produces well-resolved 2D patterns of about 800 protein spots. These gels can be analyzed for spot intensity variations between healthy and mutant samples, and proteins in affected spots can be assigned by mass spectrometry. A large decrease in the steady-state levels of two nuclear-encoded subunits of respiratory chain complex IV (cytochrome *c* oxidase) was identified in both MERRF and MELAS mutation-carrying mitochondria [117].

A detailed computer-assisted quantitative comparison of MERRF mutation-carrying and healthy mitochondria-derived 2D maps enlarged knowledge of the fate of about 800 silver-stained protein spots [P. Tryoen-Tóth et al., unpublished]. A systematic analysis of all spots in 11 gel couples allowed detection of 38 protein spots in the mutated mitochondria, altered in intensity compared with healthy ones. These include additional nuclear-encoded subunits of respiratory chain complexes as well as mitochondrial metabolic enzymes and of proteins involved in translation machinery. Most remarkably, subtle perturbations such as changes in the post-translational modification pattern of an enzyme were found. Thus, the proteomic approach delivers a global view of the status of mutated mitochondria, highlighting, beyond the direct consequences of the primary molecular impact of a mutation on the tRNA structure/function relationship, secondary events involving perturbations in mitochondrial metabolic pathways. These in turn suggest cross-talk between the mitochondrial and nuclear genomes.

Conclusion and perspectives

mt tRNA gained interest in both fundamental and medical aspects in recent years. tRNAs in health form a subgroup of RNA, with specific structural and functional rules, still not completely elucidated, which distinguish them from cytosolic human tRNAs and from 'classical' tRNAs in general. Further exploration will allow their mode of action to be delineated in detail and for them to be considered from an evolutionary point of view. The correlation between point mutations in mt tRNA genes and severe neurodegenerative disorders stimulated molecular investigations of the mitochondrial translational system. Present understanding of the primary molecular defects points to numerous events within protein synthesis, generally converging (either as an individual effect or as a combination of effects) on a decreased rate of synthesis of the 13 mitochondrial respiratory chain subunits. Since exceptions were reported in which the rate of protein synthesis was not significantly affected despite malfunctioning of the mutant tRNA [93], the contribution of additional factors to disease etiology has to be considered. Along this line, comparative proteomic investigations of healthy and mutation-carrying cells appear promising [117; P. Tryoen-Tóth et al., unpublished). Such studies have to be performed on isolated mitochondria as well as on the cytosol. Further, comparative transcriptomes of healthy and disease-carrying cells [118] will contribute to pinpoint important secondary partners involved in the cascade of events leading from the genotype to the phenotypes of mt tRNA diseases.

The major limitation in the understanding mt tRNA disorders remains access to efficient, representative model systems. While the creation of cybrid cell lines allowed for valuable comparative investigations, their derivation from cancer cells remains a drawback. Primary cell cultures (e.g. myoblasts) overcome this defect, but homoplasmy is often not achievable. Several model systems are currently explored (reviewed in [119]). *S. cerevisiae* appears as a suitable simple organism since it allows positional cloning and is presently being investigated in the context of mitochondrial disorders linked to nuclear gene mutations [120, 121]. Moreover, complete restoration of complex I activity in human cells lacking the essential mt DNA-encoded subunit ND4 has been achieved by introduction of the yeast NADH-quinone oxidoreductase gene ND1 [122]. A yeast mitochondrial transformation system has been set up for study of a tRNA mutation [123]. However, a specific drawback resides in the large structural differences existing between yeast mt tRNAs (which are of canonical type) and mammalian mt tRNAs. Thus it is likely that the structure/function relationships of yeast tRNAs are different from those of human mt tRNAs. *C. elegans*, a worm which contains structurally 'bizarre' mt tRNAs has so far only been used to model a disorder linked to a mutation in a nuclear-encoded mitochondrial protein [124]. More appropriate models such as transgenic or knockout mice for a given mt tRNA gene, in which the effects of a mutation can be studied at the level of the whole organism, are still missing. Impressive progress has been made in the field, however. In a first variety of transgenic mice, impairment of mitochondrial functions involved the abolition of nuclear-encoded genes for mitochondrial proteins [125, 126]. Further, disruption of a nuclear gene for the mitochondrial transcription factor A causes the depletion of mt DNA, loss of mitochondrial transcripts, loss of mt DNA-encoded polypeptides and severe respiratory chain deficiency [127, 128]. This system allows spatial and temporal control of the knockout and makes it possible to create respiratory chain deficiency in selected cell types/tissue/organ of the animal [126, 128]. A transmitochondrial mouse harboring a pathogenic mt DNA large deletion has also

been reported [129]. In this model, the mutant mt DNA was transmitted maternally to the germ lines, and its accumulation induced mitochondrial dysfunction in various tissues [130]. Mitochondria in mouse tissues with mt DNA deletion showed normal functions until the mt DNA deletion accumulated. Below a level of 79% it had no pathophysiological effect because of complementation by the wild-type mt DNA [131]. Another knockout study describes a transmitochondrial mouse obtained by transferring exogenous mutant mt DNA (a point mutation near the 3′ end of the 16S rRNA gene) into the mouse female germ line by means of embryonic stem cell cybrids [132]. These animal models have proven that heteroplasmic mt DNA mutations can be transmitted and will produce phenotypic abnormalities. Such exciting developments are the harbingers of a new wave of research on human mitochondrial disorders, and offer the promise of a greater understanding of the basic biology of mt tRNAs in health and disease.

Acknowledgements. We thank Louis Levinger for many stylistic improvements of the manuscript. Our work on human mt tRNAs is supported by Centre National de la Recherche Scientifique (CNRS), Université Louis Pasteur Strasbourg (ULP), Association Française contre les Myopathies (AFM) and European grant QLG2-CT-1999-00660. BS is supported by a fellowship from the Ministère de la Recherche.

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