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SURVEY AND SUMMARY

Mitochondrial tRNA 3′ end metabolism and human disease

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ABSTRACT

Over 150 mutations in the mitochondrial genome have been shown to be associated with human disease. Remarkably, two-thirds of them are found in tRNA genes, which constitute only one-tenth of the mitochondrial genome. A total of 22 tRNAs punctuate the genome and are produced together with 11 mRNAs and 2 rRNAs from long polycistronic primary transcripts with almost no spacers. Pre-tRNAs thus require precise endonucleolytic excision. Furthermore, the CCA triplet which forms the 3′ end of all tRNAs is not encoded, but must be synthesized by the CCA-adding enzyme after 3′ end cleavage. Amino acid attachment to the CCA of mature tRNA is performed by aminoacyl-tRNA synthetases, which, like the preceding processing enzymes, are nuclear-encoded and imported into mitochondria. Here, we critically review the effectiveness and reliability of evidence obtained from reactions with *in vitro* transcripts that pathogenesis-associated mutant mitochondrial tRNAs can lead to deficiencies in tRNA 3′ end metabolism (3′ end cleavage, CCA addition and aminoacylation) toward an understanding of molecular mechanisms underlying human tRNA disorders. These defects probably contribute, individually and cumulatively, to the progression of human mitochondrial diseases.

INTRODUCTION

The human mitochondrial genome encodes 13 out of more than 80 polypeptide subunits of the mitochondrial respiratory chain complexes and contains 24 additional genes required for mitochondrial protein biosynthesis (1,2). These include 2 rRNA genes and 22 tRNA genes, one for each of 18 amino acids, and 2 each for $tRNA^{Leu}$ (which read UUR and CUN codons) and $tRNA^{Ser}$ (which read UCN and AGY codons). The mitochondrial genome undergoes a 10- to 17-fold higher rate of mutation than the nuclear genome (3). Most mitochondrial mutations lead to polymorphisms (considered to be harmless), while a growing number correlate with various disorders. Since 1988 when the first pathogenesis-associated mutations in the mitochondrial genome were characterized (4,5), over 150 disease-correlated mutations have been found, about 100 of them located in tRNA genes [for a compilation, see MITOMAP: A Human Mitochondrial Genome Database 2004 (http://www.mitomap.org)]. The tRNA genes make up only ~10% of the mitochondrial genome (Figure 1A); these genes are thus 'hot-spots' for mutation-based mitochondrial pathogenesis.

The tRNA-linked disorders cover a wide range of symptoms and syndromes, including myopathies, encephalopathies, cardiopathies, deafness, ophthalmoplegia, diabetes and others. The relationship between mutations and disorders is complex; a single mutation can lead to different symptoms and different mutations to the same disorder. All these mitochondrial tRNA disorders more or less severely affect the energy metabolism of the cell. Efforts to understand the molecular mechanisms underlying their genotype/phenotype relationships have been made over the last 15 years; numerous reviews summarize the clinical and biological aspects (6–13) or the molecular mechanisms affected during the tRNA life cycles (14–18). Owing to their central function in protein synthesis, the malfunction of mutant tRNAs is thought to play a key role in the corresponding mitochondrial pathologies and has therefore been extensively investigated. Various aspects were explored, emphasizing aminoacylation properties and steady-state levels of aminoacyl-tRNA in mitochondria, but also including tRNA synthesis, structure, stability and interaction with partners of the translational machinery. However, so far only a limited number of mutations and of tRNAs have been explored, and for each, only one or a subset of possibilities were investigated. Neither general rules nor common features have emerged, and further studies are thus necessary.

Studies are complicated due to intrinsic properties of mitochondria and mitochondrial tRNAs (heteroplasmy, limited access to natural material and almost no possibility for genetic manipulation). Mitochondria are present in hundreds of copies per cell, each of them carrying more than one copy of DNA. A mitochondrial mutation can thus be heteroplasmic, varying in proportion to wild-type from 0 to 100% mutated DNA in the different tissues of an affected patient (Figure 1B). The threshold for effect of a mitochondrial mutation is usually between 15 and 50% of the normal functional level (referring to a combination of the proportion of the mutant tRNA in the

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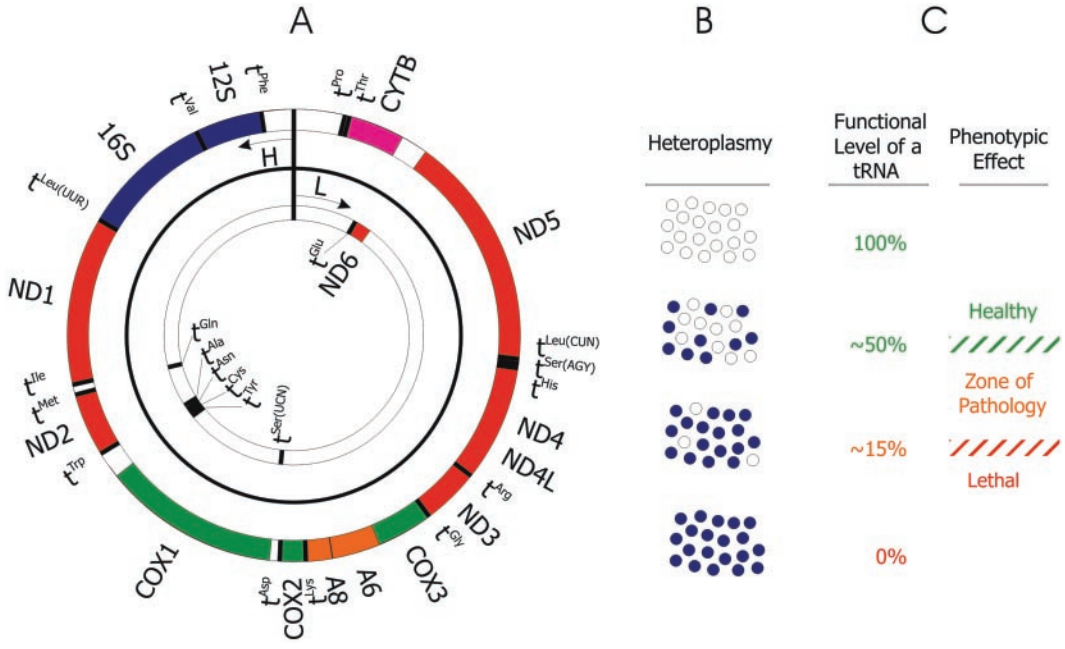

Figure 1. (**A**) Human mitochondrial gene map. H with counterclockwise arrow and L with clockwise arrow designate direction of heavy and light DNA strand transcription. Outer ring is the light transcript, and inner ring is the heavy transcript. rRNAs and mRNAs are filled in with colors. Black bars designate tRNAs which punctuate the mitochondrial genome. (**B**) Heteroplasmy. Mixed (heteroplasmic) populations of wild-type and mutant mitochondrial genomes are present. Filled circles indicate mutant mitochondrial genomes and open circles indicate wild-type. (**C**) Thresholds. The thresholds for pathology are typically between 15 and 50% of mitochondrial tRNA function, affected by the extent of heteroplasmy (B) combined with effectiveness of the mutant tRNA in the series of reactions illustrated (Figure 2), in translation, and with its stability. A lower functional level would be lethal and a higher level would be without a phenotype.

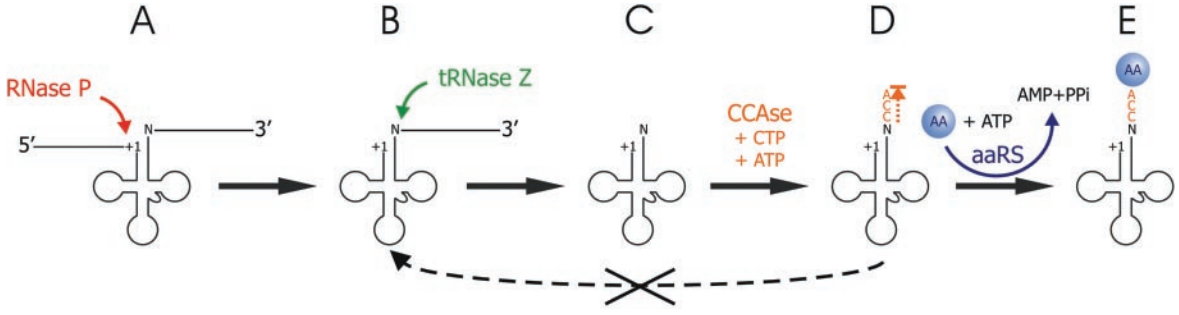

Figure 2. The tRNA end processing pathway followed by aminoacylation. (**A**) tRNA is transcribed as a precursor, with a 5′ end leader and a 3′ end trailer. (**B**) RNase P has endonucleolytically cleaved the tRNA at +1. (**C**) tRNAse Z endonucleolytically cleaves the precursor on the 3′ side of the discriminator base (N; +73). (**D**) CCA-adding enzyme (CCAse) adds CCA to the 3′ end of the tRNA (N) produced by tRNase Z cleavage. (**E**) tRNA is charged with the cognate amino acid by a specific aminoacyl-tRNA synthetase (aaRS). Dashed line from CCA in (D) to tRNAse Z between (B) and (C) with an X through it indicates that 3′-CCA of mature tRNA is a tRNase Z anti-determinant (39).

heteroplasmic population with the effect of the mutation on processing, aminoacylation and function in translation; Figure 1C). Above the upper threshold, no pathology is observed, and below the lower threshold, the condition would be lethal. The heteroplasmic state also complicates analysis and interpretation of mutational effects. To minimize this problem, cybrid cells homoplasmic for a specific mutation can be produced by fusion of enucleated patient cells with a ρ^0 cell line lacking mitochondria (19). Although such cells allowed the evaluation of several properties of mutated tRNAs (e.g. steady-state level of aminoacylation, stability and post-transcriptional modification), their construction is difficult and time consuming, so that only a handful of mutations have been analyzed so far. Moreover, the variety of cell types used to generate cybrids with different nuclear backgrounds (e.g. HeLa, osteosarcoma and lymphoblasts) does not allow straightforward comparisons (14). *Ex vivo* analyses, based on purification of a specific tRNA from cultured cells or from biopsies, are very powerful but have quantitative limitations (20,21).

The alternative approach, which avoids heteroplasmic effects in the study of pathological tRNAs and gives access to a sufficient amount of material, uses enzymes that are either recombinantly expressed or extracted from cultured cells without mitochondrial diseases, and tRNA substrates or precursors that are produced by *in vitro* transcription. This approach has recently made it possible to investigate the effects of several point mutations on various aspects of tRNA metabolism (Figure 2). Here, we review the defects in mitochondrial tRNA 3′ end metabolism revealed by *in vitro* studies, including processing (cleavage of the 3′-trailer and CCA addition) and

aminoacylation. Point mutations in mitochondrial tRNAs may contribute to tRNA malfunction by affecting the efficiency of one or a combination of these reactions. Advantages and limitations of the *in vitro* approach within the framework of investigating molecular mechanisms of human mitochondrial tRNA disorders will be discussed below.

tRNA 3′ END METABOLISM

The 22 human mitochondrial tRNAs are synthesized from polycistronic precursor transcripts where they are interspersed among 11 mRNAs and two rRNAs (2,22,23), often with no spacers (Figure 1A). Table 1 provides details on the neighborhood of each tRNA gene. In about half the cases, tRNA excision from primary transcripts leads to mature mRNAs and rRNAs with proper ends, others are either flanked by a small (1–2 nt) spacer and several even overlap by one residue with the adjacent RNA, as in the case of $tRNA^{Tyr}$/$tRNA^{Cys}$. Therefore, the processing of mitochondrial tRNAs requires precise endonucleolytic cleavage at both 5′ and 3′ ends (Figure 2). Pre-tRNA 3′ ends are subsequently completed by the addition of the CCA triplet, and a subset of nucleotides becomes post-transcriptionally modified. Finally, mature and correctly folded tRNAs are esterified at the 3′ end with the cognate amino acid by the corresponding aminoacyl-tRNA synthetase. They are subsequently carried to the ribosome by translation factor [elongation factor (EF)-Tu], allowing protein synthesis. This brief overview illustrates the importance of 3′ end metabolism for both tRNA synthesis and tRNA function.

Mutations in tRNAs may affect the reaction efficiency of the enzymes involved in tRNA 3′ end metabolism in a direct way, or indirectly via changes in structural properties of the precursor or mature tRNA. Importantly, in this context, human mitochondrial tRNAs deviate structurally from classical tRNAs (24). Most extreme is $tRNA^{Ser(AGY)}$, in which the entire D-domain is missing, and an extended anticodon stem is present. Other tRNAs possess all expected secondary structural domains and can be folded into a cloverleaf. Large variations in the size of the D-loop, and especially T-loop (which is strictly maintained at 7 nt in canonical, nuclear-encoded tRNAs), are observed, and classically conserved or semi-conserved nucleotides (e.g. GG in the D-loop, UUC converted into TΨC in the T-loop), however, are absent. These common deviations from canonical secondary structure lead, in turn, to unpredictable tertiary interactions, suggesting greater flexibility of mitochondrial tRNAs. Owing to the nucleotide composition bias of the two mitochondrial DNA strands (1), 14 tRNAs (transcribed from the heavy strand; Figure 1A) are A-rich (36%) and G-poor (14%), and eight tRNAs (transcribed from the light strand) are U-rich (31.6%) and C-poor (16.4%), weakening the helical domains of tRNA secondary structures (24). These structural peculiarities may influence all aspects of the life-cycle of human mitochondrial tRNAs and contribute to the effects of pathogenic mutations.

CLEAVAGE OF tRNA 3′-TRAILERS

tRNA end processing

Excision of tRNAs from primary polycistronic mitochondrial transcripts is catalyzed by two specialized enzymes, RNase P and tRNase Z (the tRNA 3′ end processing endonuclease, previously known as 3′-tRNase or RNase Z). Although not obligatory, a reaction order favoring cleavage by RNase P at the 5′ end of a pre-tRNA followed by tRNase Z 3′ end cleavage

Table 1. tRNA punctuation and 3′ end sequence

tRNA	Strand[a]	NT[b]	5′ Flanking gene[c]	3′ Flanking gene[c]	Note on flanking gene(s)[d]	3′ End flanking sequence[e]
Phe	H	577–674	—	12S rRNA	Contiguous	A/AAU
Val	H	1602–1670	12S rRNA	—	Contiguous	A/GCU
Leu(UUR)	H	3230–3304	16S rRNA	ND1 mRNA	Contiguous/5′ end at 3307	A/ACA
Ile	H	4263–4331	ND1 mRNA	—	Needs 1 A	A/GGA
Met	H	4402–4469	—	ND2 mRNA	Contiguous	A/AUU
Trp	H	5512–5579	ND2 mRNA	—	Contiguous	G/UAA
Asp	H	7518–7585	—	COX2 mRNA	Contiguous	A/AUG
Lys	H	8295–8364	—	ATP8 mRNA	Contiguous	A/AAU
Gly	H	9991–10058	COX3 mRNA	ND3 mRNA	Both contiguous	A/AUA
Arg	H	10405–10469	ND3 mRNA	ND4 mRNA	Needs 2 As/Contiguous	A/AUG
His	H	12138–12204	ND4 mRNA	$tRNA^{Ser(AGY)}$	Needs 1 A/Contiguous	C/GAG
Ser(AGY)	H	12207–12265	$tRNA^{His}$	$tRNA^{Leu(CUN)}$	Both contiguous	A/ACU
Leu(CUN)	H	12266–12336	$tRNA^{Ser(AGY)}$	ND5 mRNA	Both contiguous	A/AUA
Thr	H	15888–15953	—	—	—	A/AAU
Pro	L	16203–15955	—	—	—	U/UUG
Glu	L	14742–14674	—	—	—	A/AGU
Ser(UCN)	L	7516–7446	—	—	—	G/UCU
Tyr	L	5891–5826	—	$tRNA^{Cys}$	Contiguous	A/GCU
Cys	L	5826–5761	$tRNA^{Tyr}$	—	Needs 1 A	U/CUC
Asn	L	5729–5657	—	$tRNA^{Ala}$	5′ end at 5655	G/UAA
Ala	L	5655–5587	—	—	—	A/GCU
Gln	L	4400–4329	—	—	—	G/AAA

[a]H or L designates DNA strand transcribed (see Figure 1).
[b]Refers to position in the mitochondrial genome (1).
[c]Gene product encoded immediately upstream (5′ flanking gene) or downstream (3′ flanking gene) of the tRNA. In columns 4 and 5, '—' indicates no gene product encoded immediately upstream or downstream of the tRNA, respectively.
[d]States whether the flanking gene on the 5′ or 3′ side of the tRNA is contiguous, requires addition of one or two As to the 3′ end after tRNA excision or has a short spacer.
[e]3′ End sequence presented as discriminator base/following 3 nt.

(Figure 2A–C) is observed in many systems, including higher plants (25), human mitochondria (26) and budding yeast (27). While RNase P efficiently cleaves substrates with long 3′ end trailers, tRNase Z is apparently sensitive to the length of the 5′-leader (28). Experiments discussed below have therefore been performed on simplified model substrates.

Characterization of tRNase Z

Interestingly, the single fruit fly tRNase Z gene encodes both the nuclear and mitochondrial enzymes (29), and the ratio of nuclear/mitochondrial distribution in such cases is often ~20:1 (A. Hopper, personal communication). tRNase Z RNAi knockdowns cause the accumulation of both nuclear-encoded and mitochondrial pre-tRNAs with 3′ end extensions, but did not produce a phenotype or reduce the amount of mature cytoplasmic tRNA. The amount of mature mitochondrial $tRNA^{Ile}$ and $tRNA^{Tyr}$, however, was clearly reduced (29). The concentration of tRNase Z is thus more likely to be limiting in mitochondria than in nuclei, but issues of steady-state concentration and half-life of both the processing enzyme and the tRNA product must also be considered.

Human tRNase Z has also been cloned, recombinantly expressed and partially characterized, leading to the conclusion that these enzymes belong to the ELAC family (30). Both human homologs ELAC1 and ELAC2 (the short and long forms, respectively) possess the tRNase Z activity (31). It is unclear why humans and a number of other metazoans encode multiple forms of tRNase Z. Sorting servers (e.g. Mitoprot; http://ihg.gsf.de/ihg/mitoprot.html) predict that only the long form (ELAC2) carries an N-terminal mitochondrial targeting sequence, and ELAC2 has a lower K_M and a wider substrate range than ELAC1 (32), consistent with ELAC2 function as mitochondrial tRNase Z. Since the human mitochondrial form of tRNase Z has not yet been proven, however, tRNase Z activity was obtained from HeLa mitoplast extracts for the analysis of mitochondrial tRNA 3′ end processing (26,33,34).

ELAC2 was first characterized as a prostate cancer susceptibility gene (35). Two of the identified mutations which are weakly linked to prostate cancer are located in or close to the phosphodiesterase II domain, the active site of tRNase Z. ELAC2 is associated with mitotic spindles (36), and the RNAi knockdown of the *Caenorhabditis elegans* homolog of ELAC2 interferes with germline proliferation (37), but a possible function in mitochondrial tRNA processing, in which certain mutations increase the risk of human prostate cancer, is also worth considering.

CCA, a tRNase Z anti-determinant

The CCA triplet needed at the 3′ end of all mature tRNAs is not mitochondrially encoded (rightmost column in Table 1), and must thus be added to the discriminator base (the last encoded 3′ end nucleotide) by CCA-adding enzyme after 3′ end processing. Mature *in vitro* transcribed tRNA with CCA at its 3′ end is a tRNase Z anti-determinant (38,39); dashed line with an X through it; Figure 2D→B,C; [for an alternative interpretation see (40)], being neither a substrate for nor an inhibitor of tRNase Z. This ensures that mature tRNA proceeds smoothly to aminoacylation without recycling through tRNase Z. Interestingly, a partial CCA terminus consisting of –CC is sufficient to produce the anti-determinant effect.

The CCA terminus is also a tRNase Z anti-determinant in *Bacillus subtilis*, in which the CCA triplet is encoded in some tRNA genes, while it must be added post-transcriptionally to the transcripts of others. Knockout of *B.subtilis* tRNase Z shows that most of the CCA-containing pre-tRNAs are processed by a 3′-exonuclease, while those without encoded CCA are processed endonucleolytically (41). In *Thermotoga maritima*, CCA is transcriptionally encoded at the 3′ end of tRNA genes. Surprisingly, CCA is a cleavage site determinant in this organism, guiding tRNase Z to efficiently cleave downstream of the CCA triplet instead of after the discriminator base (42).

Defective tRNA end processing as a model for pathogenesis *in vivo*

The evidence which led to a model linking defective tRNA processing to pathogenesis was first obtained from studies of mitochondrial RNA in cells harboring disease-associated mutations in $tRNA^{Leu(UUR)}$ (43,44). This tRNA gene is located between the genes for 16S rRNA and the ND1 subunit (Figure 1A). There is no spacer between the 3′ end of 16S rRNA and position +1 of $tRNA^{Leu(UUR)}$, and only a 2 nt spacer is present between the discriminator base of $tRNA^{Leu(UUR)}$ and the initiation codon of the ND1 message (Table 1). An increase in the steady-state concentration of a discrete precursor (RNA 19) consisting of the linked 16S rRNA-$tRNA^{Leu(UUR)}$-ND1 RNA was observed with the A3243G and A3302G mutations (43,44), which are located in the D-loop and acceptor stem of the gene product, respectively (see Figure 3).

Reduced tRNase Z processing efficiency of pathogenesis-associated tRNA precursors

The hypothesis that pathogenesis-associated mutations could cause defective tRNA end processing, thereby contributing to disease, was further investigated *in vitro* using precursors with both 5′ and 3′ end extensions (26,33,45). We have tested the hypothesis that pathogenesis-associated mutations in tRNAs could reduce the efficiency of precursor 3′ end cleavage using wild-type and pathogenesis-associated mutations in $tRNA^{Ser(UCN)}$ (26), $tRNA^{Ile}$ (46) and $tRNA^{Leu(UUR)}$ (34) (Figure 3 and Table 2). According to this hypothesis, poor excision of tRNA could contribute to pathology. This would only be the case, however, if the intra-mitochondrial concentration of the processing enzyme is limiting.

The clearest example of a pathogenesis-associated mutation which could cause defective tRNA 3′ end processing is the T7445C substitution in $tRNA^{Ser(UCN)}$, which causes non-syndromic deafness (47,48). T7445C falls just on the 3′-side of the discriminator base of $tRNA^{Ser(UCN)}$ (Figure 3), outside of the mature tRNA, suggesting that processing reactions depending on tRNA structure or sequence would be unaffected. The mutation, however, changes 3′ end processing, leading to defective 3′ ends (47). The cell lines homoplasmic for T7445C display approximately one-third of the steady-state level of $tRNA^{Ser(UCN)}$ observed in homoplasmic wild-type sibling cell lines (48). Interestingly, the T7445C substitution changes the sequence following the discriminator base (underlined) from G/UCU– to G/CCU–, a tRNase Z anti-determinant [discussed above and in (39)]. The pathogenesis-associated T7445C tRNA precursor could not be cleaved *in vitro*, while wild-type $tRNA^{Ser(UCN)}$ could be processed

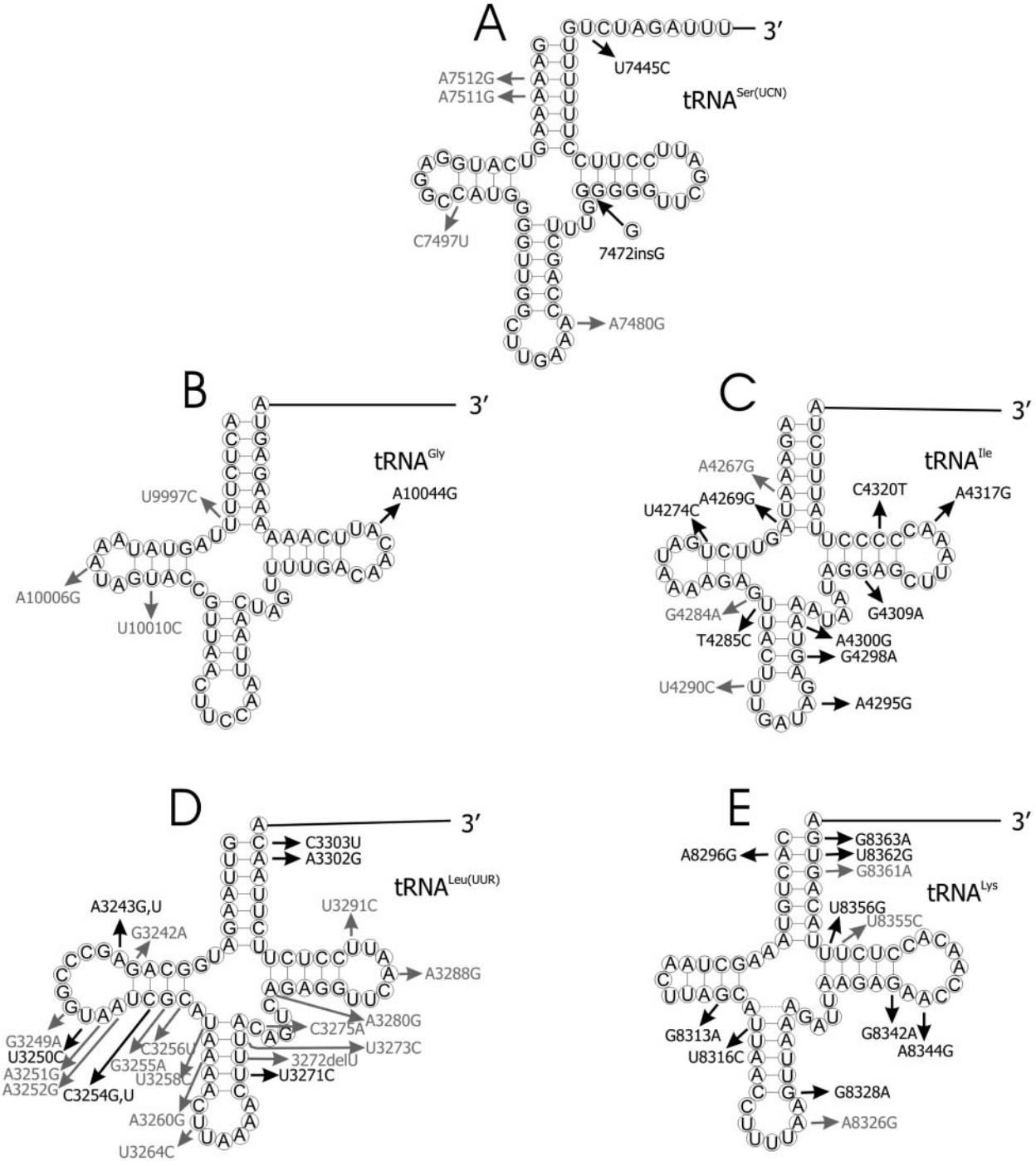

Figure 3. Mitochondrial tRNAs and pathogenesis-associated mutations analyzed for their effects on 3′ end processing, CCA addition and/or aminoacylation. (**A**) $tRNA^{Ser(UCN)}$, (**B**) $tRNA^{Gly}$, (**C**) $tRNA^{Ile}$, (**D**) $tRNA^{Leu(UUR)}$ and (**E**) $tRNA^{Lys}$. tRNAs are represented in their secondary cloverleaf structures. Mutations detected so far in the corresponding genes are indicated by arrows, and numbering is according to the mitochondrial genome (1). Black arrows and numbers refer to pathology-related mutations for which one or more of the enzymatic activities described here have been studied (see Table 2); grey numbers and arrows identify those pathology-related mutations which have not yet been characterized as to their effects on any of these reactions (MITOMAP).

efficiently (26). Additionally, $tRNA^{Ser(UCN)}$ with an extra G at the boundary between the V-loop and the T-stem (7472insG) seems to be processed and aminoacylated less efficiently than the normal tRNA (49,50). A possible rescue function for 3′-exonucleases in mitochondria [e.g. in cells with the T7445C mutation in $tRNA^{Ser(UCN)}$] has been discussed previously (26,50). In principle, any endonucleolytic cleavage which unlinks $tRNA^{Ser(UCN)}$ from the neighboring $tRNA^{Tyr}$ located ~2.5 kb downstream (Figure 1A) could be followed by 3′-exonucleolytic cleavage to the discriminator.

Among trinucleotides found at the 3′ end of human mitochondrial tRNAs following the discriminator base, /CCA is not observed, nor is /CC, and /C is found only once (in $tRNA^{Cys}$; rightmost column in Table 1). In six cases [$tRNA^{Met}$,

Table 2. Effects of mutations in human mitochondrial tRNAs on 3′ end metabolism

tRNA	Mutation Location in mitochondrial genome	Location in tRNA structural domains	Disease	Structure Effect	Reference	tRNAse Z Loss in efficiency[a]	Reference	CCAse Loss in efficiency[a]	Reference	aaRS Loss in efficiency[a]	Reference
$tRNA^{Gly}$	A10044G	T-loop	SD	Weakened D/T-loop interaction	(63)	nd		70–80%[b]	(63)	nd	
$tRNA^{Ile}$	A4269G	Acc-stem	CM	Stability decreased	(51,94)	12.5	(46)	nd		1.3	(75,76)
	T4274C	D-stem	OP	nd		4.2	(46)	nd		25	(75,76)
	G4284A		Mixed	nd		nd		nd		nd	
	T4285C	AC-stem	OP	nd		3.2	(46)			50	(75,76)
	A4295G	AC-loop	CM	No change	(75)	10.3	(46)			0.77	(74)
	G4298A	AC-stem	OP	nd		nd		nd		>1000	(75,76)
	A4300G	AC-stem	CM	nd		2.1	(46)	nd		5	
	G4309A	T-stem	OP	Local change	(46)	7.7	(46)	nd		nd	
	A4317G	T-loop	CM	Reorganization of T-stem	(63,79)	10.0	(46)	3.2–3.4	(63)	3.75	(74–76)
	C4320T	T-stem	CM	nd		4.2	(46)	nd		0.6	(75,76)
$tRNA^{Leu(UUR)}$	A3243G	D-loop	MELAS, DMDF	Dimerization of tRNA	(95)	2.2-Fold loss	(34)	0.9	(34)	2–2.5 (25 for native tRNA)	(21)
				Small rearrangement in D-stem/loop	(80)					11.8	(80)
	A3243T	D-loop	EM	Small rearrangement within the D-stem/loop	(80)	nd		nd		317	(80)
	T3250C	D-loop	MM	No change	(80)	nd		1.3	(34)	5.0	(80)
	C3254G	D-stem	MM	nd		nd		nd		18.3	(83)
	C3254T	D-stem	Polymorphism	Stabilization structure	(83)	nd		nd		0.26	(83)
										No effect	(81)
	T3271C	AC-stem	MELAS, DM	AC-stem disrupted	(82)	nd		1.3	(34)	3	(82)
				Weakening of the D-stem	(81)					No loss if combined with mutation in D-stem	(81)
										2–2.5	(21)
										3.8	(80)
	A3302G	Acc-stem	MM	nd		3.3	(34)	1	(34)	nd	
	C3303T	Acc-stem	CM, MM	nd		3.2	(34)	5.5	(34)		(80)
$tRNA^{Lys}$	A8296G	Acc-stem	DMDF, MERRF	nd		nd		nd		1.3	(79)
	G8313A	D-stem	MNGIE	nd		nd		nd		7000	(79)
	T8316C	AC-stem	MELAS	nd		nd		nd		2.0	(79)
	G8328A	AC-stem	EM	nd		nd		nd		4900	(79)
	G8342A	T-loop	PEO, MS	nd		nd		nd		1.4	(79)
	A8344G	T-stem	MERRF	No effect	(79)	nd		nd		0.3	(79)
	A8348G	T-loop	Polymorphism	nd		nd		nd		0.6	(79)
	T8356C	T-stem	MERRF	nd		nd		nd		4.0	(79)
	T8362G	Acc-stem	SM	nd		nd		nd		10	(79)
	G8363A	Acc-stem	MICM, DEAF, MERRF, LS	nd		nd		nd		1.4	(79)

Abbreviations used: Acc-stem, acceptor stem; AC-stem, anticodon stem; CM, CardioMyopathy; DM, Diabetes Mellitus; DMDF, Diabetes Mellitus, Deafness; DEAF, Deafness; EM, encephalomyopathy; LS, Leigh syndrome; MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged red fibers; MICM, maternal inherited cardiomyopathy; MM: mitochondrial myopathy; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; MS, myoclonic seizures; nd, not determined; OP, ophtalmoplegia; PEO, progressive external ophtalmoplegia; SM, skeletal myopathy.

[a]Efficiencies of reaction are measured by the ratio k_{cat}/K_M. Loss in efficiency of a mutant tRNA respective to wild-type is calculated by the ratio k_{cat}/K_M (wild-type)/k_{cat}/K_M (mutant).

[b]Percentage of decrease in rate.

$tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Arg}$ and $tRNA^{Leu(CUN)}$], the trinucleotide following the discriminator base is a mitochondrial translation initiation codon (/AUG, /AUA, /AUU or /AAU). Genes for $tRNA^{Phe}$ and $tRNA^{Thr}$, like $tRNA^{Lys}$, also have the trinucleotide AAU following the discriminator base, although the sequence immediately downstream does not encode a polypeptide in these cases. In conclusion, there is no natural tRNase Z anti-determinant in the sequences of 21 tRNA genes, and /C in $tRNA^{Cys}$ would not be expected to have much effect.

The kinetics of tRNase Z processing was performed using eight substitutions in $tRNA^{Ile}$ associated with cardiomyopathy or ophthalmoplegia (46), and three in $tRNA^{Leu(UUR)}$ correlated with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and assorted myopathies (34) (Figure 3). Four of the mutations (A4269G, A4295G, G4309A and A4317G) reduce the tRNase Z reaction efficiency up to ~10-fold, and three $tRNA^{Leu(UUR)}$ mutations decrease the efficiency up to ~3-fold relative to wild type (Table 2), all due to lower V_{max}. Interestingly, in $tRNA^{Leu(UUR)}$, the reduction in processing efficiency increases with proximity to the 3′ cleavage site (WT > A3243G > A3302G ≈ C3303U). These results support the hypothesis that 3′ end processing defects are among the molecular events involving mutant mitochondrial tRNAs which contribute to mitochondrial tRNA disorders. Note, however, that there can be discrepancies between results obtained with wild-type and mutant tRNAs from cybrid cells and those from *in vitro* transcripts when it is possible to directly compare them. For example, the $tRNA^{Ile}$ mutation A4269G was demonstrated to affect stability without any evident effect on maturation when the tRNAs were extracted from homoplasmic cybrid cells (51).

RNase P

This review is principally concerned with reactions at the 3′ end of tRNA and mitochondrial pathogenesis, but for the sake of completeness, 5′ end cleavage by RNase P should also be mentioned. Human mitochondrial RNase P activity was found by some to be indistinguishable from nuclear RNase P (52,53), while others found rat and human mitochondrial and nuclear RNase P to have different characteristics (33,54). The composition of human mitochondrial RNase P has not been completely resolved because it is difficult to purify mitochondria free of cytosolic or nuclear contamination (55). The number of RNase P molecules per mitochondrion required to process the mitochondrial transcripts could be very low (53), and if the intra-mitochondrial concentration of enzymes involved in tRNA metabolism is limiting, the modest reductions in processing efficiency arising from mutations in tRNA could lead to the accumulation of precursors and reduce the amount of functional mature tRNA.

SYNTHESIS OF THE CCA TERMINUS

Cloning, expression and characterization of tRNA nucleotidyltransferase

tRNA requires the base triplet CCA at the 3′-terminus for its aminoacylation and participation in protein synthesis. In mitochondrial tRNAs, this sequence must be added post-transcriptionally as an essential step in tRNA maturation (Figure 2). CCA addition is catalyzed by CCA-adding enzymes which are conserved in all organisms of all kingdoms. In humans, the mitochondrial, nuclear and cytosolic forms of this enzyme are encoded by a single gene located on chromosome 3 (56). A mitochondrial target sequence encoded in exon 1 ensures delivery of a proportion of the CCA-adding enzyme to mitochondria. Importantly, both the tRNase Z and CCA-adding enzyme are encoded in both forms by using two or three in-frame start codons separated by a short distance, with the mitochondrial targeting sequence being present only when translation initiates at the upstream AUG. Since CCA-adding enzymes are closely related to poly(A) polymerases, these enzymes were sometimes mistakenly identified by sequence analysis (57,58). The recombinant enzymes, however, can easily be distinguished based on their biochemical activity. The human CCA-adding enzyme was therefore cloned, recombinantly expressed and characterized (56,59). While showing kinetic properties comparable to those of other CCA-adding enzymes, the human enzyme has a specific affinity for mitochondrial tRNAs which lack some of the canonical elements, such as the TΨC-loop. The N-terminal domain of this enzyme contains the catalytic core as well as features such as nucleotide recognition/discrimination and specificity for tRNA (60,61). Recent domain swap experiments between the CCA-adding enzyme and poly(A) polymerase indicate that C-terminal domains also contribute to the discriminatory power of CCA addition (62).

Influence of pathology-related mutations on CCA addition

The activity of CCA-adding enzyme can be influenced by changes in sequence and/or structure of substrate tRNAs which can consequently contribute to pathogenicity, as first reported in certain disease-associated mutations in mitochondrial $tRNA^{Ile}$ and $tRNA^{Gly}$ (63) as well as $tRNA^{Leu(UUR)}$ (34). In both $tRNA^{Ile}$ and $tRNA^{Gly}$, an A was replaced by G in the TΨC-loop (A4317G in $tRNA^{Ile}$ and A10044G in $tRNA^{Gly}$; Figure 3). These two domains combine to form the 'elbow' of the L-shaped three-dimensional (3D) structure of tRNA, a recognition element for CCA-adding enzymes (64–67). Structure probing experiments on *in vitro* transcripts carrying these replacements revealed that they lead to an aberrantly stable TΨC-arm or to a weakened interaction between the TΨC- and D-loop, respectively (63). In the kinetic analysis, K_M values were only slightly affected, indicating that the enzyme's affinity for the mutated tRNAs was not reduced. The observed decrease in k_{cat} suggests that the chemical step of catalysis is affected, possibly due to an inappropriate orientation of the tRNA substrate in the catalytic core of the enzyme (63). Besides $tRNA^{Ile}$ and $tRNA^{Gly}$, five pathogenic mutations in $tRNA^{Leu(UUR)}$ were investigated for their effects on CCA addition (Figure 3) (34). Only the substitution closest to the 3′-terminus, C3303T (causing cardiomyopathy), showed a significant effect on CCA addition (Table 2), reducing nucleotide incorporation by a factor of 5.5. While V_{max} was unaffected, K_M increased, indicating that C3303T reduces the affinity of the CCA-adding enzyme for tRNA without interfering with catalysis.

These examples show that pathogenesis-associated mutations which affect the same enzymatic reaction can do so by different means, either interfering with catalysis (as in A4317G in $tRNA^{Ile}$ and A10044G in $tRNA^{Gly}$) or affecting the strength of enzyme–substrate interaction [C3303T in $tRNALeu^{(UUR)}$]. Furthermore, the same mutation [C3303T in $tRNA^{Leu(UUR)}$] affects two different enzymes differently, causing a decrease in the V_{max} of tRNase Z and an increase in the K_M of CCA-adding enzyme (34).

AMINOACYLATION

Once the tRNA is synthesized (i.e. the steps of 5′ and 3′ end cleavage and CCA addition are finished as in Figure 2A→D, post-transcriptional modifications are complete and 3D folding has taken place), the amino acid can be attached by the cognate aminoacyl-tRNA synthetase (aaRS; Figure 2D→E). It can then be brought to the ribosome by transient binding to translation factors, allowing transfer of the amino acid to the growing polypeptide chain, thereby fulfilling its fundamental role in protein synthesis.

Mitochondrial aminoacyl-tRNA synthetases

Eukaryotic cells contain at least two sets of aminoacyl-tRNA synthetases, one devoted to cytoplasmic protein synthesis, the other to translation of the mitochondrial genetic information. In humans, cytosolic and mitochondrial enzymes are generally encoded by distinct genes, with the exception of GlyRS and LysRS [(68); C. Florentz, unpublished]. Enzymes targeted to mitochondria are synthesized with a mitochondrial signal sequence which is cleaved after import. Interestingly, mitochondrial synthetases recognize not only mitochondrial but also cytosolic tRNAs and tRNAs from other organisms; on the other hand, mitochondrial tRNAs are not recognized by non-cognate enzymes (69), probably due to their unusual structural properties.

Specific recognition of a tRNA by its cognate aaRS is based on the presence of 'identity elements', usually a set of bases or base pairs in the tRNA. Such elements have been defined precisely for a large number of bacterial, archaeal and lower eukaryotic tRNAs (70,71) but remain largely unknown for human mitochondrial tRNAs. Efficient aminoacylation also depends on structural features within the tRNA and on more global conformational properties.

Mutations in mitochondrial tRNAs reduce aminoacylation efficiency

To evaluate the impact of the mutations in pathology-related tRNAs on aminoacylation, wild-type and mutant mitochondrial tRNAs have been prepared by *in vitro* transcription and aminoacylated by the corresponding recombinantly expressed aminoacyl-tRNA synthetases. The first aminoacylations tested in such an *in vitro* system were those specific for serine and isoleucine. The serine system contributed to a fundamental understanding of tRNA/aaRS recognition, but pathology-related mutations were not analyzed (72,73). In contrast, 10 pathology-related mutations within $tRNA^{Ile}$ associated with either ophthalmoplegia or cardiomyopathy were tested in the presence of a crude mitochondrial enzymatic extract (74) or with cloned and over-expressed IleRS (75,76) (Figure 3). Some mutations strongly reduce the ability of the tRNA to be aminoacylated, while others have only a very limited effect (Table 2). Mutations correlated with ophthalmoplegia reduced the isoleucylation efficiencies (as measured by the ratio k_{cat}/K_M compared to wild-type) from between 25- and 50-fold to more than 1000-fold. More modest reductions in aminoacylation efficiencies (or no effect) were reported for mutations correlated with cardiomyopathies (75,76). Mutants with compensatory mutations that restore disrupted base pairing regain wild-type aminoacylability. The structural effects of the mutations thus provide a stronger explanation for the reductions in aminoacylation efficiency than direct interference in tRNA/synthetase recognition. The analysis of $tRNA^{Ile}$ variants also revealed that a mutant tRNA can potentially compete for the corresponding wild-type tRNA by inhibiting its aminoacylation. This situation could mimic the *in vivo* case, in which wild-type and mutant tRNAs co-exist in heteroplasmic patient cells (Figure 1B).

The use of *in vitro* transcripts leads to difficulties for investigation of aminoacylation properties of several human mitochondrial tRNAs other than $tRNA^{Ile}$ and $tRNA^{Ser}$ because the corresponding wild-type transcripts do not fold into the expected cloverleaf secondary structures. The cases of $tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$ are of particular interest.

Wild-type $tRNA^{Lys}$ folds into an extended hairpin (77) due to the absence of a particular post-transcriptional modification, the N1-methyl group on adenine 9 (78). This methyl group prevents base pairing of nucleotides 9 [position 8303 in the mitochondrial genome, (see Figure 3)] and 64 (position 8355) from producing an extended acceptor stem in native $tRNA^{Lys}$. The role of this methyl group has been mimicked in an *in vitro* transcript by mutating either nucleotide 9 or 64 to prevent them from base pairing (77). The corresponding 'pseudo-wild-type' transcripts fold into the cloverleaf structure and become active in aminoacylation with kinetic properties close to those of cytoplasmic human $tRNA^{Lys}$ (79). By second-site mutation of such 'pseudo-wild-type' transcripts, it became possible to investigate the effect on aminoacylation of all pathology-related mutations described so far in the mitochondrial $tRNA^{Lys}$ gene (79). Two (G8313A and G8328A) out of the eleven pathology-related mutations lead to a very strong loss (5000- to 7000-fold) in aminoacylation efficiency (k_{cat}/K_M); the other mutations had mild (2- to 10-fold losses in efficiency) or no effects. Thus, as in the case of $tRNA^{Ile}$, only a subset of mutations affects aminoacylation. The mechanisms by which the G8313A and G8328A mutations affect lysylation are unknown. Both mutations are located in the anticodon branch (anticodon domain and D-domain) on the same side on a model L-shaped 3D tRNA structure where they could directly interfere with LysRS interaction (79), while mutations without effect are located on the opposite side or in the amino acid accepting branch. Alternatively, these mutations could cause large structural rearrangements of the tRNA, leading indirectly to failure of synthetase recognition.

$tRNA^{Leu(UUR)}$ has also been prepared by *in vitro* transcription. The cloned and purified human mitochondrial LeuRS accepts this transcript as a substrate at a 16- to 50-fold lower efficiency than native $tRNA^{Leu(UUR)}$ extracted from cell lines (21,80). Here, the transcript only folds partially into a tRNA structure, despite the presence of sequence elements typical of classical tRNAs required for correct folding.

Indeed, the amino acid acceptor stem and the TΨC-arm fold normally, but a large 'floppy' domain is observed instead of well-defined D and anticodon domains (34,80,81). Footprinting experiments suggest that the synthetase induces a structural change within the tRNA, leading to a productive interaction. Several mutations were analyzed to determine their effects on leucylation (Figure 3 and Table 2). For example, the effects of MELAS mutation A3243G and a second mutation at the same position correlated with encephalomyopathy (A3243T) lead to different effects (21,80). While mutation A3243G displays ~10-fold reduced efficiency of aminoacylation, A3243T leads to a 300-fold decrease. The T3271C substitution in the anticodon stem has only a 3-fold negative effect, which can be reversed by a compensatory mutation (82). As was found for $tRNA^{Ile}$ and $tRNA^{Lys}$, mutations can affect aminoacylation efficiency either through k_{cat} or through K_M. Local structural perturbations were observed between some pathogenesis-associated mutations and wild-type $tRNA^{Leu(UUR)}$ (80,82).

The structurally unorganized domain in $tRNA^{Leu(UUR)}$ prepared by *in vitro* transcription, as opposed to the well-folded native tRNA extracted from cell lines (83), can be stabilized so as to more closely resemble a canonical structure. The site-directed mutagenesis that replaces C•A mismatches in either the D-stem or the anticodon stem by G-C pairs leads to cloverleaf structures, thus improving aminoacylation efficiency (81,83). These 'pseudo-wild-types' represent valuable reference molecules for a systematic investigation of pathology-related mutations on $tRNA^{Leu(UUR)}$ functions.

CHANGE IN 3′ END METABOLISM OF MITOCHONDRIAL tRNAs IS AN IMPORTANT CONSEQUENCE OF MUTATION

Pathology-related mutations affect 3′ end metabolism

Analysis of transcribed wild-type or engineered 'pseudo-wild-type' substrates (tRNAs and tRNA precursors), or mutated derivatives, allow the effects of mutations to be screened *in vitro*. Mutations were found to affect any combination of the activities involved with 3′ end metabolism of tRNAs (Figure 2B–E): cleavage of the 3′-trailer, CCA addition and aminoacylation. Although data summarized in Table 2 are not yet complete, it is clear that for many mutations, at least one of the three activities is significantly affected, making the 3′ end of tRNAs a reasonable place to look for the effect of pathogenesis-associated mutations. Only three mutations have been tested for their effect on all three functions. Among these, mutation A3243G in $tRNA^{Leu(UUR)}$ leads to a weak effect on 3′ end cleavage (2.2-fold decrease in efficiency), no effect on CCA addition and a mild effect on aminoacylation (2- to 12-fold decrease in efficiency). The other two, A4317G in $tRNA^{Ile}$ and C3303T in $tRNA^{Leu(UUR)}$, substantially reduce the efficiency of 3′ end cleavage and CCA addition but have little or no effect on aminoacylation. Nine mutations in $tRNA^{Ile}$ have been tested for their effect on both 3′ end cleavage and aminoacylation. For some of them (4269, 4295 and 4320), cleavage of the 3′ end trailer of the primary tRNA transcript is reduced but aminoacylation is not affected; for others (4274, 4284 and 4300), 3′ end maturation is unaffected, but reduced efficiency of aminoacylation is observed.

Whatever the affected activity, the end result is expected to be the same, a decrease in aminoacyl-tRNA available for protein synthesis. While the effect can differ at every level, an accumulation of small effects on several aspects of tRNA metabolism could lead to a strong global negative effect, sufficient to place the level of mitochondrial protein synthesis within the zone of pathology (Figure 1C). Indeed, limiting the amount of aminoacyl-tRNA, by any means, reduces the rate of translation (84,85). Such reductions have been observed *in vivo* in many cases involving pathogenesis-associated mitochondrial tRNAs (49,86–89). For some mutations, the 3′ end metabolism was not affected (see Table 2); in these cases, other levels are presumably responsible for pathology, as suggested by the results of *in vitro* and *in vivo* studies by numerous investigators [for example see (17,18) and references therein]. The defects were observed in tRNA synthesis and maturation, including impaired termination of transcription, impaired RNase P processing and absence of post-transcriptional modifications (especially of the anticodon triplet, hindering codon reading). Post-aminoacylation functions of tRNA, including interaction with EF-Tu, codon reading or polysome formation, were sometimes affected. Decreased stability is the most common feature of mutant mitochondrial tRNAs associated with pathogenesis.

Structural aspects

As discussed above, the mechanisms by which mutations in tRNA affect the enzymatic activities of tRNA metabolism remain largely unknown. Mutations may interfere directly with recognition by the concerned enzyme, or change the overall structure enough to perturb the recognition. Owing to the intrinsic structural weakness of human mitochondrial tRNAs, the effect of mutations on tRNA structure may be amplified. Most of the pathology-associated mutations are conservative nucleotide transitions (90). When found in stems, canonical base pairs are often changed to weak G-U (U-G) wobble pairs or to C•A (A•C) mismatches (see Figure 3). In many cases, the effect on stem structure would be expected to be mild, because G-U and C•A can hydrogen bond with strength comparable to the A-U pair (91). Their structural effect could be magnified, however, since they are often found in regions which are already thermodynamically weak (18). Further illustrating this point, A4309G in the T-stem of $tRNA^{Ile}$ displays a weakened secondary structure (46), and T3271C in the anticodon stem of $tRNA^{Leu(UUR)}$ loses its negative effect on aminoacylation when combined with a stabilizing substitution in the D-stem (82).

EFFECTIVENESS AND CAVEATES CONCERNING USE OF *IN VITRO* TRANSCRIPTS AS TOOLS FOR SYSTEMATIC SCREENING

The results summarized here on the potential impact of mitochondrial tRNA mutations on 3′ end metabolism were obtained using *in vitro* transcribed substrate tRNAs or tRNA precursors. The *in vitro* strategy of RNA preparation is of particular interest for human mitochondrial tRNAs since it allows the preparation of a large amount of any tRNA (either wild-type or mutant). Furthermore, *in vitro* kinetic analysis can lead to mechanistic insight. The structural flexibility of

these tRNAs leads, in some cases, to the need to create 'pseudo-wild-type' molecules which have a single mutation introduced to stabilize structure and allow correct folding. Thoroughly documented in at least two cases [$tRNA^{Lys}$ and $tRNA^{Leu(UUR)}$] to react in a manner comparable to that of the corresponding natural tRNAs extracted from cells, we believe they are relevant substitutes which allow useful analyses of second-site (pathogenesis-associated) mutations.

The lack of post-transcriptional modifications can be a drawback since such modifications can affect the structure and function of tRNA (92). Post-transcriptional modifications in classical tRNAs play either of two major roles, stabilizing tRNA structure and/or providing a recognition element for interacting partners such as aminoacyl-tRNA synthetases or mRNA codons. Therefore, aminoacylation properties of *in vitro* transcripts may be different from those of fully modified tRNAs *in vivo*. However, mutations introduced into the 'pseudo-wild-type' molecules are designed to stabilize the tRNA structure, replacing the structural role of the naturally occurring modifications to the extent that is feasible and allowing for efficient aminoacylation.

The *in vitro* approach is also valid when testing 3′ end maturation activities such as tRNase Z activity, because this enzyme processes primary transcripts which may not yet be modified. Concerning CCA addition, this reaction can take place before all modifications are introduced into the tRNA (93). CCA-adding enzyme requires a properly folded tRNA acceptor stem to synthesize the CCA triplet; this domain has been shown to be correctly folded in the case of *in vitro* transcripts which have been analyzed for CCA addition [$tRNA^{Ile}$, $tRNA^{Gly}$ and $tRNA^{Leu(UUR)}$]. Modified nucleotides may thus be unnecessary for CCA addition and by inference, for the preceding tRNase Z reaction.

Significance of small effects of mutations on enzyme activities measured *in vitro*

Two major questions affect the interpretation of measured kinetic values. (i) To what extent do *in vitro* effects reflect the *in vivo* situation? (ii) Do small effects on reaction efficiency contribute to the expression of disorders? The first question is difficult to answer since *in vitro* assays take into account neither possible competition events nor the cellular environment, which the *in vivo* approach does. Moreover, one approach measures kinetic parameters of an enzymatic reaction leading to a value for *efficiency*, while the other measures the *steady-state level* of reaction products (e.g. aminoacyl-tRNA). These parameters cannot be compared directly, leading to apparent discrepancies between some of the *in vitro* and *in vivo* results. A valuable approach would be to measure reaction kinetics using *in vitro* wild-type and mutated transcripts and corresponding native wild-type and mutated tRNAs extracted from cybrid cell lines. In the case of $tRNA^{Lys}$, results were the same (20,79). In the case of $tRNA^{Leu(UUR)}$, however, results were significantly different (21). The native mutated $tRNA^{Leu(UUR)}$ (A3243G) showed a 25-fold loss in aminoacylation efficiency when compared to the corresponding wild-type tRNA, while the mutated *in vitro* transcript only led to a 2.5- to 3-fold loss compared to the wild-type transcript. The transcripts can thus underestimate the impact of mutations; they can nonetheless usefully serve as indicators/detectors of the effects of mutation on aminoacylation.

Concerning the magnitude of effects and their relevance to pathology, Table 2 shows between 2.1- and 12.5-fold effects of mutations on cleavage of the 3′-trailer and at most a 5.5-fold decrease in efficiency of CCA addition. On the other hand, effects on aminoacylation properties of transcripts can be much greater, ranging from a 2-fold to a several 1000-fold decrease in efficiency. While several 1000-fold reductions in aminoacylation would be expected to contribute strongly to pathogenicity, even small reductions in individual maturation steps can contribute to the onset of disease, amplified by the cumulative effects of these impairments. Along these lines, the A4269G mutation in $tRNA^{Ile}$ displays a 2-fold decrease in affinity for EF-Tu (94).

FUTURE PROSPECTS

Pathogenesis-associated tRNAs may contribute to mitochondrial disease due to individual effects or to accumulation of a number of deficiencies in end maturation, including 3′ end cleavage by tRNase Z and CCA addition by tRNA nucleotidyltransferase, and in aminoacylation. Effects of the pathogenesis-associated substitutions on these reactions, analyzed using *in vitro* transcripts, does not preclude other events, including 5′ end processing by RNase P, post-transcriptional modifications, elongation factor binding, ribosome binding, etc.

One promising direction is the analysis of nuclear-encoded enzymes involved in mitochondrial tRNA metabolism. In the case of enzymes encoded by single genes which are targeted to the mitochondria, cytoplasm and/or nucleus, it will be important to establish the relative enzyme concentrations in the individual compartments, which will greatly influence tRNA metabolism.

Virtually all pathogenesis-associated tRNA mutations can be investigated using *in vitro* transcripts. It will thus be possible to quantify and classify the effect(s) of individual mutations on metabolism as it relates to pathogenicity and to accumulate fundamental information concerning structure/function relations in human mitochondrial tRNAs. Although the *in vitro* strategy is not universally applicable, it is a valuable approach for unraveling many aspects of tRNA-based mitochondrial pathology before undertaking more costly *in vivo* analyses.

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