Impairment of tRNA processing by point mutations in mitochondrial tRNA$^{Leu(UUR)}$ associated with mitochondrial diseases

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Abstract Several point mutations in mitochondrial tRNA genes have been linked to distinct clinical subgroups of mitochondrial diseases. A particularly large number of different mutations is found in the tRNA$^{Leu(UUR)}$ gene. We show that base substitutions at nucleotide position 3256, 3260, and 3271 of the mitochondrial genome, located in the D and anticodon stem of this tRNA, and mutation 3243 changing a base involved in a tertiary interaction, significantly impair the processing of the tRNA precursor in vitro. In correlation with other studies, our results suggest that inefficient processing of certain mutant variants of mitochondrial tRNA$^{Leu(UUR)}$ is a primary molecular impairment leading to mitochondrial dysfunction and consequently to disease.

Key words: tRNA processing; RNase P; Mitochondrial disease; Mutation

1. Introduction

Mitochondrial diseases are a diverse group of clinical conditions defined by mitochondrial dysfunction [1]. Hence, although essentially multisystemic, they are predominantly manifested as encephalomyopathies. In the past years several mutations (mainly base substitutions, large scale deletions and duplications) in mtDNA have been linked to distinct clinical subgroups of mitochondrial encephalomyopathies and thereby pushed research on the pathogenesis of this heterogeneous group of disorders (for reviews, see [2–5]). tRNA genes appear to be particularly frequently affected by such mutations. However, the actual molecular consequences of these base substitutions with respect to tRNA structure and function are still largely unclear, and so is the molecular pathology. Since in vivo studies are complicated by a varying degree of heteroplasmy, variable nuclear genetic background, and limited by the availability of biopsy material, most approaches have used a cell culture system designed for the analysis of mtDNA mutations to study the molecular pathology of the associated diseases [6–8]. These studies revealed that tRNA base substitutions cause a generalized impairment of mitochondrial protein synthesis and consequently a decrease in oxidative phosphorylation capacity [7–14]. More specifically, in one case a decreased aminoacylation of the affected tRNA was found [15], and in two other cases the specific accumulation of the tRNA precursor [8,16]. Furthermore, in some cases the steady-state level of the affected tRNA was decreased [12–15]. Yet, complementing research, using in vitro systems with their inherent advantage to directly address potential underlying molecular mechanisms, has not been carried out.

The recent development of a human mitochondrial in vitro tRNA processing system that faithfully reflects the in vivo processing event enables to analyze the effect of mutations in tRNA genes on this critical step in mitochondrial biogenesis [17]. Notably, mammalian mitochondrial tRNAs are made together with rRNA and mRNA genes as large polycistronic primary transcripts. Within these, the tRNAs act as recognition signals for endonucleases, RNase P and 3′ pre-tRNase, cleaving precisely at the 5′ and 3′ ends of the tRNAs [17], and thus, due to the unique genetic arrangement [18], releasing tRNAs, rRNAs, and mRNAs as a natural consequence of tRNA processing [19]. Alterations, qualitatively or quantitatively, will therefore not only affect the respective tRNA, but also the immediately adjacent RNAs and moreover generate unusual (levels of) precursor RNA species. In this paper the processing efficiency and specificity of wild-type and mutant, disease-associated precursors of (mt)tRNA$^{Leu(UUR)}$ are compared using the aforementioned in vitro system. Whilst none of the 12 base substitutions studied changes the specificity of the processing reaction, a severe decrease in the processing rate of certain mutant precursors was observed. The quantitative results of 5′ end processing by mtRNase P are furthermore compared to the data obtained for the same mutant tRNA precursors with nRNase P, and provide novel aspects for the understanding of RNase P substrate recognition.

2. Materials and methods

2.1. Precursor tRNA substrates

phL, the template for in vitro transcription of wild-type (mt)pre-tRNA$^{Leu(UUR)}$, has previously been described in detail [17]. The different point-mutant substrate templates were derived from phL by PCR mutagenesis essentially as described [20]. The sequence of the complete transcribed region of each plasmid was confirmed by dideoxy sequencing (T7 Sequencing kit, Pharmacia). Plasmids were cleaved with restriction endonuclease *Rsa*I prior to transcription with bacteriophage T3 RNA polymerase. In vitro transcription reactions contained 80 mM HEPES-KOH (pH 7.6), 12 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol, 500 μM each of ATP, CTP, and UTP, 150 mM GTP, 5 μCi [α-^{32}P]GTP to achieve the desired specific activity, 1 unit/μl rRNasin (Promega), 5 units/ml yeast inorganic pyrophosphatase (Sigma), 1 unit/μl RNA polymerase, and 50 μg/ml template DNA. Transcripts were purified by denaturing polyacrylamide gel electrophoresis.

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Abbreviations: 3′ pre-tRNase, precursor-tRNA 3′-endonuclease; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; (mt), mitochondrial; mtDNA, mitochondrial DNA; mtRNase P, mitochondrial RNase P; nRNase P, nuclear RNase P; PEG, polyethylene glycol

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2.2. Preparation of tRNA processing enzymes

Mitochondrial tRNA processing activities (mtRNase P and mt3′ pre-tRNase) were prepared by PEG precipitation and glycerol gradient sedimentation as recently described [21].

nRNase P was prepared as previously described [17].

2.3. Processing assays

Processing reactions were carried out as previously described [17]. The substrate concentration was 10 nM. Enzymes and substrate were preincubated separately under reaction conditions. The reactions were started by mixing enzyme and substrate, and aliquots were withdrawn at different times. Reactions were stopped and analyzed by denaturing polyacrylamide gel electrophoresis as previously described [17]. Unprocessed precursor and processing products were quantitated from polyacrylamide gels using a PhosphorImager and ImageQuant software (Molecular Dynamics). The relative initial cleavage rates of different substrates (product formation per given time interval relative to product formation from wild-type substrate) were deduced from linear ranges of curves.

3. Results

3.1. Point mutations in (mt)tRNALeu(UUR)

Eleven base substitution mutations within the (mt)tRNA$^{Leu(UUR)}$ gene have been reported to date. With 10 of these associated with mitochondrial disease, the (mt)tRNA$^{Leu(UUR)}$ gene not only exhibits the largest number of presumably pathogenic mutations within a localized region of the mtDNA, but also roughly as many mutations as the remaining 21 tRNA genes together. Fig. 1 and Table 1 display all these mutations and their position in the mitochondrial genome [18], and this position number will be used throughout this report when referring to a specific mutation. (Note: while this study was in progress, two further mutations associated with mitochondrial encephalomyopathy, the deletion of nucleotide 3272 and an A to T transversion at position 3243, were reported [22,23].)

The base substitutions were introduced in a previously described precursor for (mt)tRNA$^{Leu(UUR)}$ [17] by in vitro mutagenesis. Sequencing of the mutagenized template plasmids ensured that the in vitro transcribed precursors did not contain any other mutation than the desired one. Fig. 1 schematically displays the tRNA precursor and all the base substitutions. In addition to the 11 mutations described, an A at position 3266 was replaced by a G as a further control (Fig. 1, Table 1). This substitution changes the anticodon from UAA to UGA, normally found in (mt)tRNA$^{Ser(UCN)}$. A similar anticodon swap in (mt)tRNA^{Pro} has been reported in a patient with mitochondrial myopathy [24].

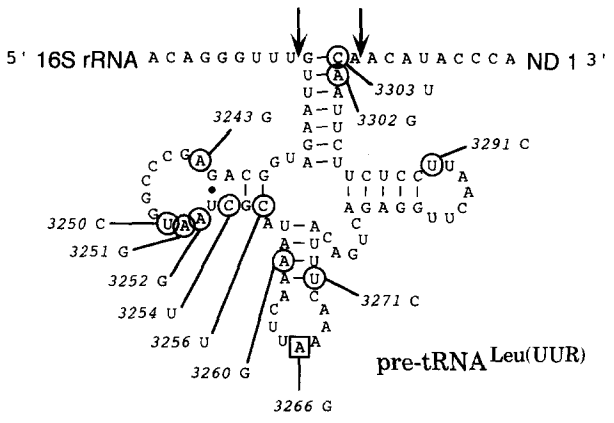

Fig. 1. Schematic display of the precursor for mitochondrial tRNA$^{Leu(UUR)}$. The sequence of (mt)tRNA$^{Leu(UUR)}$ is embedded between the 16S rRNA and the NADH dehydrogenase subunit 1 mRNA (ND 1) in the primary transcript. The in vitro transcribed precursor starts with 35 nucleotides of polylinker sequence, followed by the 12 3′ terminal nucleotides of 16S rRNA, the tRNA$^{Leu(UUR)}$, and terminates at an *RsaI* restriction site 34 nucleotides downstream, within the ND 1 mRNA [17]. The 11 reported transitions are within circles (see also Table 1), and identified by their nucleotide position in the mitochondrial genome [18]. The A to G transition in the anticodon, included in this study as a control, is within a box. Arrows mark the exact cleavage sites.

3.2. Processing of mutant tRNA precursors by mitochondrial enzymes

The different mutant tRNA precursors were analyzed in parallel to the wild-type using a previously characterized in vitro processing system [17]. Crude mitochondrial extracts (data not shown) as well as partially purified mtRNase P and mt3′ pre-tRNase were employed (Fig. 2, Table 1), though the relative cleavage rates did not change upon purification of

Table 1
Processing of mutant precursors for mitochondrial tRNA$^{Leu(UUR)}$

Nucleotide position in mtDNA (tRNA)[a]	Base substitution	Key reference	Associated clinical phenotype[b] (remarks)	Relative efficiency of tRNA formation[c]	Relative processing efficiency (mtRNase P)[d]	Relative processing efficiency (nRNase P)[e]
3230–3304	none	[18]	wild-type	100	100	100
3243 (14)	A→G	[48,49]	MELAS, PEO, diabetes	19±5	19±9	76±2
3250 (20)	T→C	[50]	myopathy	68±5	63±6	88±22
3251 (20a)	A→G	[51]	myopathy	42±6	53±7	86±11
3252 (21)	A→G	[52]	encephalomyopathy	38±4	47±3	138±28
3254 (23)	C→T	[53]	? (natural polymorphism)	83±13	92±8	50±6
3256 (25)	C→T	[46]	multisystem, MELAS	not determined	4±4	119±21
3260 (29)	A→G	[54]	cardiomyopathy, myopathy	29±1	28±3	164±25
3266 (35)	A→G		? (control, anticodon)	89±6	94±8	116±8
3271 (40)	T→C	[55]	MELAS	20±6	16±4	138±6
3291 (60)	T→C	[56]	MELAS	74±3	79±4	193±19
3302 (71)	A→G	[40]	myopathy	65±6	74±4	119±4
3303 (72)	C→T	[57]	cardiomyopathy	66±5	71±4	217±9

[a]According to [18] and [58], respectively.
[b]MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; PEO, progressive external ophthalmoplegia.
[c](%) relative to wild-type, determined as described in Section 2; mean±S.D. (3–5 experiments).
[d](%) release of 5′ leader relative to wild-type, determined as described in Section 2; mean±S.D. (4–6 experiments).
[e](%) release of 5′ leader relative to wild-type, determined as described in Section 2; mean±S.D. (3 experiments).

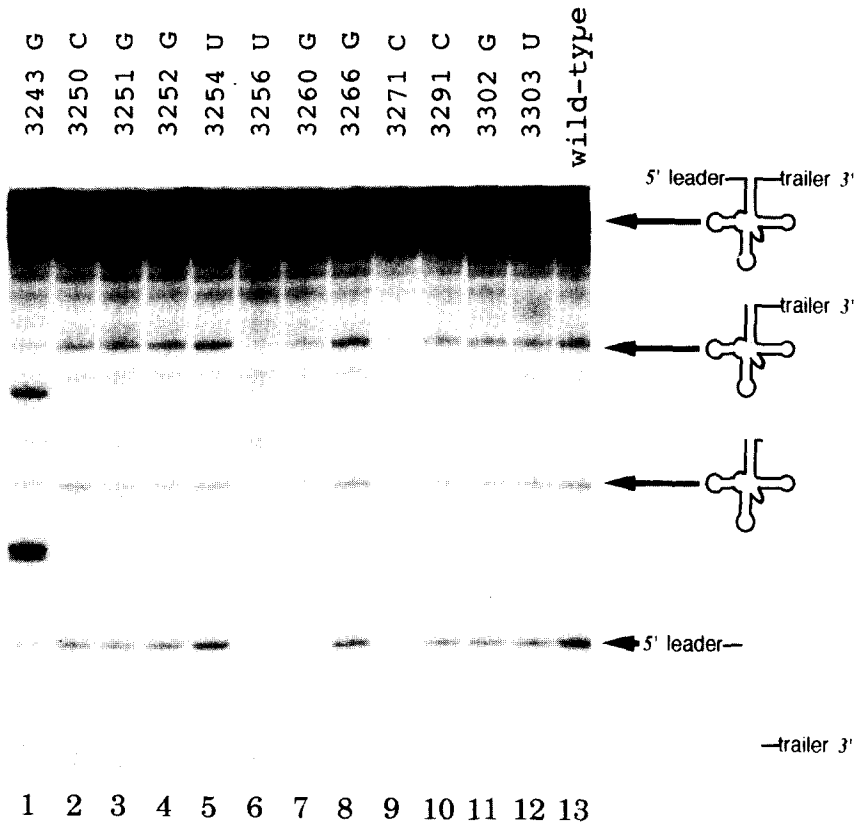

Fig. 2. Processing of mutant tRNA precursors by mitochondrial enzymes. The wild-type precursor for (mt)tRNA$^{Leu(UUR)}$ and the mutant derivatives, as indicated above each lane (compare Fig. 1 and Table 1 for identification), were incubated in parallel with mtRNase P and (mt)pre-tRNA 3'-endonuclease under the previously described conditions [17]. The cleavage products were analyzed by denaturing polyacrylamide gel electrophoresis (6% gel) and autoradiography. An early kinetic time-point is shown (6 min). Substrate and cleavage products are schematically drawn on the right. The trailer product is not visible on this exposure because of its relative lower labeling intensity. Note: The two additional RNA species of 61 and 95 nucleotides in length, present only in lane 1 (mutant 3243), are not due to aberrant processing, but were consistently observed in all batches of this very substrate, even after successive rounds of gel purification of the substrate RNA. The significance of these breakdown products is not known, nor has it been investigated. They are, however, neither dependent on nor affected by the addition of mitochondrial enzyme preparations to the substrate RNA, but are present from the very beginning in the substrate preparation, i.e. their size and amount are not detectably altered while cleavage reactions are carried out.

the enzymes. The mitochondrial enzyme preparations were furthermore verified not to contain any contaminating nRNase P using the *Escherichia coli* pre-tRNA$^{Tyr}su_3^+$ substrate [17] and by RNase protection analysis for H1 RNA, the RNA component of nRNase P (data not shown).

No qualitative processing alterations were observed with any of the studied mutant tRNA precursors. None of the 12 base substitutions affected cleavage site selection of either mtRNase P (5′ cleavage) or mt3′ pre-tRNase (3′ cleavage); the respective cleavage products of mutant substrates had exactly the same size as those of the wild-type (Fig. 2). However, considerable alterations in the processing rates of distinct mutant tRNA precursors were observed (Table 1, Fig. 2). Hence, the relative efficiency of tRNA formation and the relative processing efficiency at the tRNA 5′ end (cleavage by mtRNase P) were quantitatively determined (Table 1). The C to T transition 3256, at the base of the D stem, had the most striking effect on processing, as the cleavage rate by mtRNase P was decreased more than 20-fold, and did not allow to determine the efficiency of tRNA formation. Base substitutions at 3243, 3260, and 3271 also showed a pronounced (approximately fivefold) decrease in their ability to serve as a substrate for mtRNase P, and the relative efficiency of tRNA formation was reduced roughly to the same extent with these mutants. Yet, the remaining mutants (3250, 3251, 3252, 3291, 3302, 3303) did not markedly affect susceptibility to cleavage by mtRNase P, or overall tRNA formation. Reduction in processing efficiency was either approximately twofold (3250, 3251, 3252), or less (3291, 3302, 3303). Mutant precursors 3254, presumably a neutral polymorphism, and 3266, an A to G transition in the anticodon, were cleaved at a rate similar to that of the wild-type.

Taken together, these results demonstrate that certain disease associated point mutations in the human (mt)tRNA$^{Leu(UUR)}$ gene have a profound quantitative effect on the processing of the tRNA precursor, but do not lead to aberrant processing products. In addition to their pathobiological relevance these data may furthermore point to specific structural features of (mt)tRNAs involved in recognition by mitochondrial tRNA processing enzymes (see below).

3.3. *Processing of mutant mitochondrial tRNA precursors by nuclear RNase P*

Apart from the first studies concerning substrate specificity of human mtRNase P [17], no data about substrate recognition by vertebrate mitochondrial tRNA processing enzymes were available as yet. By way of contrast, substrate recognition by vertebrate nRNase P has been extensively studied in

recent years [25–28]. Cleavage analysis of the described mutant (mt)tRNA precursors by nRNase P, although likely of no relevance for the in vivo/in mitochondrion situation, should thus help to evaluate the results obtained with mtRNase P as well as to understand the commonalities and diversities of these two cellular RNase P enzymes.

Experiments were carried out essentially in the same way as with mtRNase P. Again, the cleavage site was not changed (data not shown). But in sharp contrast to mtRNase P, nRNase P was not markedly affected in its ability to cleave any of the mutant substrate RNAs (Table 1). Cleavage rates ranged from a twofold decrease to a twofold increase relative to the wild-type substrate. Still, there was no correlation in these alterations to those obtained with mtRNase P, thereby confirming the previous notion that substrate requirements of mtRNase P and nRNase P are different [17,29].

4. Discussion

4.1. tRNA structure and tRNA processing

Mutation 3254, presumably a neutral polymorphism, generates an A-U base pair found in other vertebrates at this position. This 'strengthening' of the D stem does not alter the processing rate of the precursor. Similarly, changing the anticodon of a tRNA (control mutation 3266) is not expected to affect tRNA structure and processing. Mutations in (mt)tRNA$^{Leu(UUR)}$ that have a pronounced effect on the processing of its precursor, on the other hand, change bases conserved throughout vertebrate evolution (3243, 3256, 3260) and/or likely involved in stabilizing a stem structure (3256, 3260, 3271) or tertiary conformation (3243). In this context it is important to note that the D and anticodon stem of (mt)tRNA$^{Leu(UUR)}$ appear to be rather weakly stabilized structures, and might thus be more prone to alterations leading to structural disturbances. The anticodon stem is composed of four A-U base pairs and a terminal A C mismatch (Fig. 1). Mutation 3271 introduces a second A C mismatch, and A to G transition 3260 generates the weaker G·U base interaction. Both have a considerable quantitative effect on processing, but that of the more severe alteration (3271) is more pronounced.

The D stem of (mt)tRNA$^{Leu(UUR)}$, although formed at its base by two G-C base pairs, is closed against the loop by a G·U following an unpaired A C (Fig. 1). Mutation 3256, which has the most striking effect on the processing rate, generates a G·U instead of the first G-C and might thereby significantly weaken the D stem. The T and acceptor stem of (mt)tRNA$^{Leu(UUR)}$ are, in contrast, stabilized by standard 'Watson-Crick' base pairing over their entire length. This might explain why mutation 3302 and 3303, both of which generate a non-canonical G·U base pair, have only a very moderate effect on precursor processing.

The A3243 at tRNA position 14, an invariant nucleotide among bacterial and cytoplasmic tRNAs [30], is hydrogen bonded to U8 according to the X-ray crystal structure of yeast tRNA^{Phe}, and contributes to the stabilization of tRNA tertiary structure [31]. This nucleotide pair is present in all known vertebrate (mt)tRNAs$^{Leu(UUR)}$, though some other (mt)tRNAs apparently lack this very tertiary interaction [30,32]. The frequently found A to G transition 3243 may nevertheless significantly affect the three-dimensional structure of (mt)tRNA$^{Leu(UUR)}$, the vertebrate (mt)tRNA most similar to 'classical' tRNA structures [18,30], and hence the processing of its precursor.

The remaining base substitutions (3250, 3251, 3252, 3291), which do not extraordinarily alter processing rates, concern nucleotides not conserved in vertebrate evolution, and, except for 3252, have not previously been implicated with higher order tRNA structure. A3252 at position 21 in the tRNA is involved in a triple interaction with the aforementioned U8-A14 base pair in the yeast tRNA^{Phe} model [30,31].

The overall rate of tRNA formation in vitro for all the different mutants follows more or less the relative cleavage rate at the 5′ end. Nevertheless this does not allow us to conclude that 3′ end processing is not affected by the mutations, since this study was not designed to analyze a possible quantitative effect on 3′ end cleavage. The results obtained for 5′ end processing by mtRNase P, however, can be compared to the data obtained with nRNase P. Interestingly, the structural alterations caused by the studied mutations are restricted to the D domain and the anticodon stem, both apparently more important for substrate recognition and cleavage by mtRNase P than by nRNase P. The recent identification of the unique processing pathway of human (mt)tRNA$^{Ser(AGY)}$, a tRNA with an abnormal anticodon stem and lacking a D domain, previously indicated the critical importance of both features for processing by mtRNase P [29]. For catalysis by human nRNase P on the other hand, the length of the acceptor stem as well as the structure of the T domain have previously been identified as critical elements [25,26]. While the latter is unlikely to play any role in mitochondrial tRNA processing, since the T domain is the least conserved part of mitochondrial tRNAs [30,32,33], a role of the conserved 7 bp acceptor stem for mtRNase P processing, although reasonable, remains to be established.

4.2. tRNA processing and mitochondrial disease

Studies in bacterial tRNA processing have shown that for a given tRNA precursor, mutations generally have similar effects in vivo as in vitro [34–36]. However, when comparing an in vitro analysis of mitochondrial tRNA processing to data obtained in vivo, one is inevitably faced with the complexities of the mitochondrial genetic system: a mixture of wild-type and mutant genomes (heteroplasmy) and variable nuclear genetic background. These problems can be partially avoided by the use of transmitochondrial cybrids [6–8], and this technology has helped to establish the causal relationship of certain (mt)tRNA$^{Leu(UUR)}$ mutations (3243, 3256, 3260, 3271) and the pathological mitochondrial phenotype [8–11,14].

Steady-state levels of (mt)tRNA$^{Leu(UUR)}$, as well as of the flanking 16S rRNA and ND1 mRNA, have been investigated in three of these mutant cell lines (3243, 3256, 3271) [8,14,16,37,38] and in tissue samples of eight MELAS-3243 patients [39]. While no significant alterations of size and amount of these RNAs were detected in cybrids harboring the 3243 or 3271 mutation, cell lines as well as patient tissues (3243) contained increased amounts of unprocessed (mt)tRNA$^{Leu(UUR)}$. Accumulation of this precursor, corresponding to 16S rRNA-tRNA$^{Leu(UUR)}$-ND1 mRNA and termed RNA 19 [8], was somewhat more pronounced in 3271 cybrids than in 3243 [16], well in correspondence with the difference of the in vitro processing rate (this study). Yet, a similar analysis of C to T transition 3256, which has the most striking effect on processing in vitro, showed only a very

slight increase in the abundance of RNA 19, but significantly reduced levels of (mt)tRNA$^{Leu(UUR)}$ and ND1 mRNA [14]. It must, however, be noted that although mutant cybrid clones may approach homoplasmy, they generally still show considerable phenotypic heterogeneity [6,8,14,16].

RNA analysis from tissues of the patient with mutation 3302 is somewhat at odds with our in vitro analysis. While this mutation has no marked effect on processing in vitro, accumulation of RNA 19 in a tissue specific manner was found in this patient [40]. It is currently not possible to resolve this discrepancy. No information on mitochondrial RNAs is available for the remaining (mt)tRNA$^{Leu(UUR)}$ mutations and the damaging effect to mitochondrial function by mutation 3250, 3251, 3252, 3291, 3302, and 3303, in any case, still awaits direct demonstration by use of, e.g. transmitochondrial cybrid technology.

The work presented in this paper demonstrates for the first time that certain mutations in the human (mt)tRNA$^{Leu(UUR)}$ gene (3243, 3256, 3260, 3271) interfere directly with the efficient processing of the tRNA precursor. This may explain the aberrant levels of certain RNA species found in the respective cybrids. Taken together the findings suggest that a processing deficiency of (mt)tRNA$^{Leu(UUR)}$ is a primary molecular impairment leading to mitochondrial dysfunction and consequently to disease. The implications of a reduced processing efficiency for mitochondrial protein biosynthesis are, however, currently only poorly understood and represent an area for future research that may lead to a more complete understanding of the molecular pathology of the associated diseases. Mitochondria could either be deprived of adequate levels of the affected RNAs (16S rRNA, tRNA$^{Leu(UUR)}$, ND1 mRNA) or the accumulating precursor (RNA 19) might act in a hitherto unknown dominant negative way. In this context it has been proposed that unprocessed 16S rRNA (RNA 19) might be incorporated into mitochondrial ribosomes and interfere with proper mitochondrial translation ('ribosome stalling' model) [38,41].

In any case, it cannot be excluded that mutations in (mt)tRNA$^{Leu(UUR)}$ impairing tRNA processing may interfere with other mitochondrial functions as well. A transcription termination factor binding site is embedded in the (mt)tRNA$^{Leu(UUR)}$ gene [42], and mutation 3243 has been shown to dramatically reduce 16S rRNA transcription termination in vitro [43,44]. However, no evidence for such an impairment has been found in transmitochondrial cybrids [8,9] or in vivo [45]. Other mutations within or very close to the binding site (3250, 3251, 3252, 3256, 3260, 3271) did furthermore not alter in vitro termination efficiency [44]. Base substitutions could nevertheless conceivably impact on one of the other steps in tRNA maturation, stability of the tRNA, and/or charging of the tRNA by its cognate aminoacyl-tRNA synthetase.

4.3. The (mt)tRNA$^{Leu(UUR)}$ hot spot

Due to the unusually large number of presumably pathogenic point mutations in (mt)tRNA$^{Leu(UUR)}$, its gene has been postulated to be an etiologic hot spot [46]. It has furthermore been suggested that higher demands on correct sequence in this gene reduce the number of potential neutral mutations [47]. Alternatively, there might be special constraints on higher order tRNA structure. In this regard (mt)tRNA$^{Leu(UUR)}$ is without doubt a highly unusual (mt)tRNA, since unlike other (mt)tRNAs it has retained all the structural features typically found in bacterial or cytoplasmic tRNAs [18,30]. It could thus be more prone to minor structural deviations that interfere with processing by enzymes tailored to recognize the broad range of (mt)tRNAs.

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