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Molecular Pathological Mechanisms of Mitochondrial tRNA Point Mutations

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original communications, which are referred to in the text by their Roman numerals I-III. In addition, some unpublished results are included.

I. **Toompuu M**, Tiranti V, Zeviani M and Jacobs HT (1999): Molecular phenotype of the np 7472 deafness-associated mitochondrial mutation in osteosarcoma cell cybrids. Hum Mol Genet 8: 2275-2283.

II. **Toompuu M**, Yasukawa T, Suzuki T, Hakkinen T, Spelbrink JN, Watanabe K and Jacobs HT (2002): The 7472insC mitochondrial DNA mutation impairs the synthesis and extent of aminoacylation of tRNA$^{Ser(UCN)}$ but not its structure or rate of turnover. J Biol Chem 277: 22240-22250.

III. **Toompuu M**, Levinger LL, Nadal A, Gomez J and Jacobs HT (2004): The 7472insC mtDNA mutation impairs 5′ and 3′ processing of tRNA$^{Ser(UCN)}$. Biochem Biophys Res Commun 322: 803-813.

ABBREVIATIONS

aa-tRNA	aminoacylated tRNA
aaRS	aminoacyl-tRNA synthetase
(e.g. SerRS	seryl-tRNA synthetase)
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumine
bp	base pair
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cDNA	complementary DNA
COX	cytochrome c oxidase
cpm	counts per minute
CTP	cytidine triphosphate
dCTP	deoxycytidine triphosphate
D-loop	displacement loop
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eEF1A	eukaryotic elongation factor 1A
EF-G	elongation factor G
EF-Ts	elongation factor Ts
EF-Tu	elongation factor Tu
ELISA	enzyme-linked immunoassay
EtBr	ethidium bromide
FAD	flavin adenine dinucleotide
FeS	iron-sulphur protein
FMN	flavin mononucleotide
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HIV	human immunodeficiency virus
HSP	heavy strand promoter
H-strand	heavy strand
i^6A	N^6-isopentenyladenosine
kb	kilobase

KSS	Kearns-Sayre syndrome
LHON	Leber hereditary optic neuropathy
LSP	light strand promoter
L-strand	light strand
LSTU	light strand transcription unit
m^1A	1-methyladenosine
m^1G	1-methylguanosine
m^2G	N^2-methylguanosine
m^3C	3-methylcytidine
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy and ragged-red fibers
M-MLV	Moloney murine leukemia virus
mRNA	messenger RNA
ms^2i^6A	2-methylthio-N^6-isopentenyladenosine
mt	mitochondrial
mtDNA	mitochondrial DNA
mtSSB	mitochondrial single-stranded binding protein
mTERF	mitochondrial transcription termination factor
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NaAc	sodium acetate
NADH	reduced nicotinamide adenine dinucleotide
NARP	neuropathy, ataxia and retinitis pigmentosa
ncDNA	nuclear DNA
np	nucleotide pair
nt	nucleotide
O_H	H-strand replication origin
O_L	L-strand replication origin
ORF	open reading frame
OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
PAP	poly(A) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEO	progressive external opthalmoplegia
PMSF	phenylmethylsulphonyl fluoride
PNPase	polynucleotide phosphorylase
pre-tRNA	precursor tRNA
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
ROS	reactive oxygen species
RT	reverse transcription
RTase	reverse transcriptase

RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
snoRNA	small nucleolar RNA
SNP	single nucleotide polymorphism
snRNA	small nuclear RNA
T	ribothymidine
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
TFAM	mitochondrial transcription factor A
tko	*technical knockout, Drosophila* gene
TLC	thin layer chromatography
tRNA	transfer RNA
ts	temperature-sensitive
u	unit
UTP	uridine triphosphate
X-gal	5-bromo-4-chloro-3-indolyl-β-$_D$-galactoside
ψ	pseudouridine
2D	two-dimensional
3D	three-dimensional

ABSTRACT

Mitochondrial DNA mutations are increasingly recognized as a cause of human disease, point mutations in mitochondrial tRNA genes being the largest group among them. The 7472insC mutation in tRNA$^{Ser(UCN)}$, for example, is associated mainly with sensorineural deafness or, in some cases, with a wider neurological syndrome.

The aim of my study was to characterize the molecular phenotype and the molecular mechanism of the 7472insC mutation. As a model, osteosarcoma cybrid cells carrying 100% mutant mtDNA were compared with control cells bearing 100% wild-type mtDNA. The main effect of the mutation was a 65% decrease in the steady-state level of the mutant tRNA$^{Ser(UCN)}$. The functional level of the mutant tRNA was even lower, due to a 25% decrease in the extent of its aminoacylation *in vivo*. The drop in the amount of the mutant tRNA was associated with only a mild, quantitative effect on mitochondrial protein synthesis in cultured cells, which was exacerbated in the presence of doxycycline, a drug which inhibits the elongation step on bacterial-type ribosomes (i.e. including those of mitochondria). The mutation also had only a modest effect on the ability of cells to grow under conditions of respiratory stress, imposed by the use of galactose in place of glucose in the culture medium, which was only impaired in combination with a decreased mtDNA copy number.

The synthesis of the mutant tRNA$^{Ser(UCN)}$ was significantly impaired, to an extent comparable with the decrease in its steady-state level. In contrast, the half-life of the mutant tRNA molecule was not decreased, and the overexpression of EF-Tu had no effect on the low steady-state level of the mutant tRNA. Based on structural analysis, the effect of the mutation on the tRNA$^{Ser(UCN)}$ molecule was also minimal, causing no difference in the pattern of base modifications or tertiary structure.

To define the precise mechanism of the mutation further, I analysed its effect on pre-tRNA processing *in vivo* and *in vitro*. tRNA$^{Ser(UCN)}$ isolated from cybrid cells carrying the 7472insC mutation was found to have been misprocessed at a high frequency *in vivo*, either at 5´-, 3´-, or both termini. The mutant pre-tRNA$^{Ser(UCN)}$ construct was also less efficiently processed at both 5´- and 3´-ends by partially purified processing enzymes *in vitro*. In the case of 3´-end processing, the mutant substrate was poorly processed only if it retained a 5´-leader, and 3´-misprocessing further impaired 5´-processing. This indicates that the 7472insC mutation can impair tRNA$^{Ser(UCN)}$ synthesis by affecting several RNA processing steps, but that the end result is highly dependent on the processing pathway used *in vivo*. This suggests an explanation for the tissue

specificity of its effects, since the processing pathway of mitochondrial pre-tRNAs appears to differ between cell-types.

The results of my study suggest that defective tRNA processing could be a potential mechanism of many other pathogenic mtDNA mutations, and one of the factors determining the tissue specificity of the diseases they cause.

In addition, I found evidence for a specific mechanism of degradation of aberrant mitochondrial tRNAs, involving 3´-polyadenylation, which could be a common pathway for mitochondrial tRNA quality control.

1. INTRODUCTION

Mitochondria play a central role in energy production by eukaryotic cells. These organelles possess their own genome encoding several essential subunits of the respiratory chain and the components of their translation machinery, including a full set of tRNAs and two rRNAs. Since 1988, mutations in mtDNA have been identified as the underlying cause of a large group of disorders, predominantly targeting tissues highly dependent on energy, such as skeletal muscle and the nervous system. Among them, tRNA point mutations are the most frequent cause of mtDNA disorders, with a great variety of clinical manifestations. Although several of these diseases correlate with the affected tRNA, the association between the mutations and the pathologies they cause remains extremely complex. At present, only a few tRNA mutations have been characterized in detail, and their molecular mechanisms are poorly understood in general. Establishing the precise pathogenic mechanisms of tRNA point mutations is an essential step towards the development of possible treatment strategies, which are currently lacking.

The 7472insC mutation in tRNA$^{Ser(UCN)}$ is associated with sensorineural deafness and, in some patients, a wider neurological syndrome. As a result of my studies, I propose a molecular pathogenic mechanism of this mutation, which could be common also to cases of other tRNA point mutations. I summarize other possible mechanisms contributing to pathogenesis, emphasizing the importance of a correct tRNA structure for its function and biogenesis. In addition, I discuss some factors that might contribute to phenotypic expression of deafness mutations in tRNA$^{Ser(UCN)}$. Finally, I provide the first evidence of a polyadenylation-dependent degradation pathway for defective tRNAs in human mitochondria, which I compare with similar mechanisms of RNA quality control in other systems.

2. REVIEW OF LITERATURE

2.1 The mitochondrion

2.1.1 Organization and function

Mitochondria are organelles that produce energy in the form of ATP as their main function. Other roles of mitochondria are diverse and include fatty acid oxidation, amino acid metabolism, biosynthesis of FeS clusters, calcium and iron homeostasis and regulation of apoptosis. Some of these functions can be considered a consequence of the endosymbiotic origin of mitochondria. For example, the apoptotic machinery may initially have been used by the bacterial invader against the host, to favour its own survival under stressful conditions (Frade and Michaelidis, 1997). Later on in the evolution of multicellular eukaryotes this machinery may have become adapted to bring about programmed cell death, involving also co-evolved eukaryotic proteins (Frade and Michaelidis, 1997; Koonin and Aravind, 2002). Biosynthesis of FeS clusters is suggested to have been crucial for the establishment and retention of endosymbiosis and might be a unifying function of mitochondria and mitochondrion-related organelles, such as mitosomes and hydrogenosomes (van der Giezen and Tovar, 2005).

Mitochondria are surrounded by a highly specialized double membrane that creates the internal matrix and the intermembrane space. The outer membrane is permeable to all molecules ≤ 5000 Da due to its non-specific pores. The inner membrane has specific features essential for oxidative phosphorylation. This membrane coupling electron transport from reducing equivalents to proton pumping is non-permeable to ions including protons, creating an electrochemical proton gradient used as the driving force for ATP synthesis. The surface of the inner membrane is vastly increased by folding into cristae with the number and morphology depending on the energy demands of the cell (Scheffler, 1999). The two membranes can form transient contacts that are suggested to create a channel for protein import, via cooperation of the independent translocation elements of the two membranes (Schülke et al, 1997; Scheffler, 1999). Three complexes are involved in protein import: the translocase of the outer membrane (TOM complex) transports all precursor proteins destined for import to the matrix, inner membrane or intermembrane space; two translocases of the inner membrane are specialized, respectively, for transport of cleavable preproteins (TIM23 complex)

or hydrophobic membrane proteins (TIM22 complex) (see review by Pfanner and Wiedmann, 2002).

Mitochondria form a dynamic network that is regulated by two opposing processes, fusion and fission. In yeast, a complex of three proteins, Fzo1p, Mgm1p and Ugo1p, serves to coordinate a two-step fusion of outer and inner membranes (reviewed by Meeusen and Nunnari, 2005). In mammals, the fusion is promoted by Fzo1p orthologs, Mfn1 and Mfn2, and Mgm1p ortholog, OPA1 (Chen and Chan, 2005). The relative levels of Mfn1 and Mfn2 could regulate mitochondrial fusion in a cell-specific way (Meeusen and Nunnari, 2005). The dynamic network is believed to control the essential functions of mitochondria, such as respiration and apoptosis (Chen and Chan, 2005) and to affect mtDNA stability (Chen and Butow, 2005).

The morphological properties of mitochondria depend on the cell type and on the physiological state of the cell. Although fusion and fission may affect mitochondrial morphology, several proteins were identified in yeast that control mitochondrial shape by a so far unknown mechanism, possibly involving contacts between the outer and inner membranes or association with the cytoskeleton (Jensen, 2005).

2.1.2 The respiratory chain
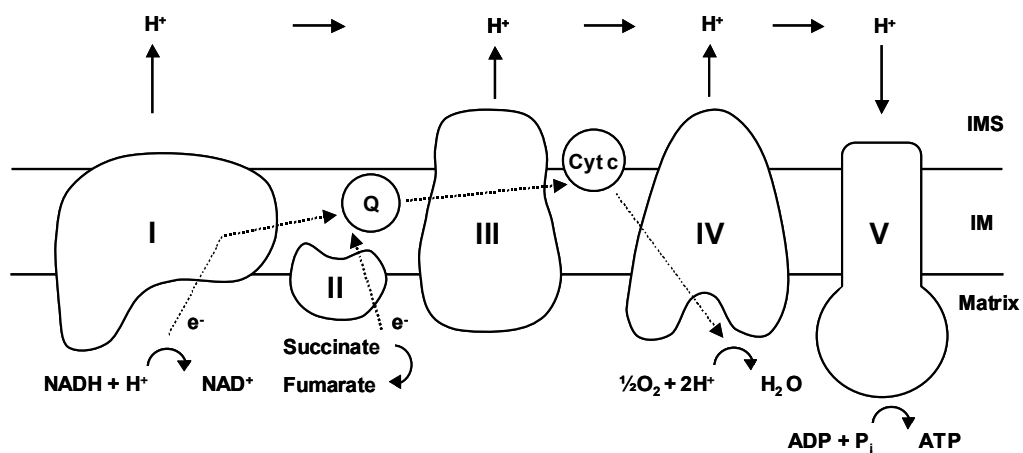
Figure 2.1. *Organization of the mitochondrial respiratory chain. Electrons (e⁻) are transferred from complexes I and II, involving complex III, to complex IV, which donates them to oxygen. Ubiquinone (Q) and cytochrome c (Cyt c) serve as the mobile carriers. Electron transfer from complexes I, III and IV is accompanied by proton (H⁺) translocation from the matrix across the inner membrane (IM) into the inner membrane space (IMS). The resulting electrochemical proton gradient is used for ATP synthesis by complex V.*

The mitochondrial respiratory chain is composed of four enzyme complexes located in the inner membrane that function as a proton pump. It is organized in order of increasing redox potential at each step of electron transfer from NADH

to O_2. Electron transfer is coupled to proton translocation across the inner membrane, and the resulting transmembrane electrochemical potential difference is converted into chemical energy in the form of ATP by ATP synthase (complex V) (Fig. 2.1).

Complex I (NADH:ubiquinone oxidoreductase) is the largest complex of the respiratory chain, consisting of 14 core subunits forming an L-shaped structure in the membrane. It catalyses the reduction of ubiquinone by NADH using FMN and several FeS clusters as cofactors. The mechanism of energy transfer by complex I is unknown and might exploit the reverse Q-cycle or the [4Fe-4S] cluster known as cluster N2 (Hirst, 2005).

Complex II (succinate:ubiquinone oxidoreductase) consists of four protein subunits, two of which function as succinate dehydrogenase in the Krebs cycle, plus five prosthetic groups. Complex II links the oxidation of succinate to fumarate at FAD, involving three FeS clusters and *b*-type cytochrome, to the reduction of ubiquinone. Complex II only translocates electrons without proton pumping. The electron transfer to heme *b* is proposed to play a role in reducing ROS formation, and is essential for aerobic respiration, whereas anaerobic organisms use the more efficient and closely related enzyme fumarate reductase, with a different organization of redox centers (Yankovskaya et al, 2003).

Complex III (cytochrome bc_1 complex) from eukaryotic organisms is a homodimer with three main catalytic subunits in each monomer carrying cytochrome *b*, cytochrome c_1 and the Rieske iron-sulfur protein. Complex III catalyses electron transfer from a membrane-bound ubiquinol to soluble cytochrome *c* by means of the protonmotive Q-cycle (reviewed by Hunte et al, 2003). As a result of redox reactions on opposite sites of the membrane, protons are translocated across the membrane as hydrogens on the ubiquinol.

Complex IV (cytochrome *c* oxidase) from eukaryotic organisms consists of 11-13 subunits, three of which are encoded by mtDNA, forming a highly conserved functional core (reviewed by Khalimonchuk and Rödel, 2005). This complex contains two hemes, *a* and a_3, and two copper centers, CuA and CuB. Complex IV catalyses the terminal reduction of O_2 to H_2O coupled to proton translocation across the inner membrane.

Complex V (ATP synthase) is composed of two parts: the membrane-embedded F_O domain and the catalytic F_1 ATPase located in the matrix. F_1 ATPase is composed of five subunits: α, β, γ, δ, and ε. The ATP synthase works via a unique rotary mechanism that produces ATP by phosphorylation of ADP using the proton gradient (reviewed by Capaldi and Aggeler, 2002). Subunits γ and ε form a rotary stalk that is bound to the *c* subunit ring of F_O at the bottom and interacts with α and β subunits at the top. Reversible rotation of subunit γ causes conformational changes of catalytic sites that results in synthesis or hydrolysis of ATP (reviewed by Boyer, 2000). Under de-energized conditions, ATP hydrolysis is prevented by binding of MgADP that blocks rotation. Recently, another mechanism was hypothesised in which rotation of subunit γ determines the conformation of subunit ε, which has an extended form in the

ADP-inhibited state, proposed to act as a safety switch (Feniouk and Junge, 2005).

Respiratory chain complexes have been observed to be associated in supramolecular 'respirasomes' with the advantage of direct substrate channelling to the enzymes and sequestration of reactive intermediates (reviewed by Schägger, 2002). In human mitochondria, the respirasome cores are formed by stable interaction of complex I with complex III, to which complex IV can also bind (Schägger et al, 2004). This association may have a specific role in the assembly and stability of complex I (Schägger et al, 2004). The ATP synthase was shown to form dimers with involvement of subunits *e* and *g*, which are not essential for enzymatic activity (Arnold et al, 1998). The dimerization is proposed to be involved in the control of the morphology of mitochondrial cristae (Paumard et al, 2002). Recently, the supramolecular organization of ATP synthase has been suggested to regulate the bioenergetic properties of mitochondria by an indirect control of the membrane potential (Bornhovd et al, 2006).

2.1.3 The human mitochondrial genome

The human mitochondrial DNA (mtDNA) is a circle of 16.6 kb that encodes 13 polypeptides, 2 rRNAs (12S and 16S) and 22 tRNAs (Fig. 2.2). The 13 proteins are components of the respiratory chain, including 7 subunits of complex I (ND1, 2, 3, 4, 4L, 5, 6), the cytochrome *b* subunit of complex III, 3 subunits of complex IV (COXI, II, III) and 2 subunits of ATP synthase (A6, A8). The two strands of mtDNA are called heavy (H) and light (L) due to their different GT content, which gives them distinct buoyant densities. The structure of mtDNA is compact, with only a 1.1 kb noncoding region including the triplex structure known as the D-loop, and containing the proposed origin of the H-strand replication (O_H), the promoters for H-and L-strand transcription (HSP and LSP) and several conserved elements possibly involved in regulation of mtDNA replication.

MtDNA is associated with proteins forming complexes called nucleoids that may be considered as units of mtDNA inheritance. Human mtDNA-associated proteins include mitochondrial transcription factor A (TFAM), the 5´-3´ DNA helicase Twinkle, DNA polymerase γ and mitochondrial single-stranded binding protein (mtSSB) (Garrido et al, 2003; Chen and Butow, 2005). Human cells contain several hundreds of nucleoids with 2-8 mtDNA molecules each (Legros et al, 2004).

The mode of mtDNA replication remains debatable. According to the traditional strand-displacement model (Clayton, 1982), replication starts from the H-strand and proceeds about two-thirds of the mtDNA length when the L-strand synthesis begins in the opposite direction. An alternative model suggests a synchronous leading- and lagging-strand synthesis that could coexist with the orthodox mode of replication (Holt et al, 2000). According to the revised model,

initially bidirectional θ replication starts from multiple origins downstream of O_H and after fork arrest proximally to O_H proceeds unidirectionally (Bowmaker et al, 2003). The replication machinery has been proposed to include RNase MRP (Chang and Clayton, 1987a; Lee and Clayton, 1998), involved in RNA primer formation and DNA polymerase γ which probably requires many other factors for its function, such as Twinkle, mtSSB, TFAM, topoisomerases and mitochondrial DNA ligase (see Fernandez-Silva et al, 2003).
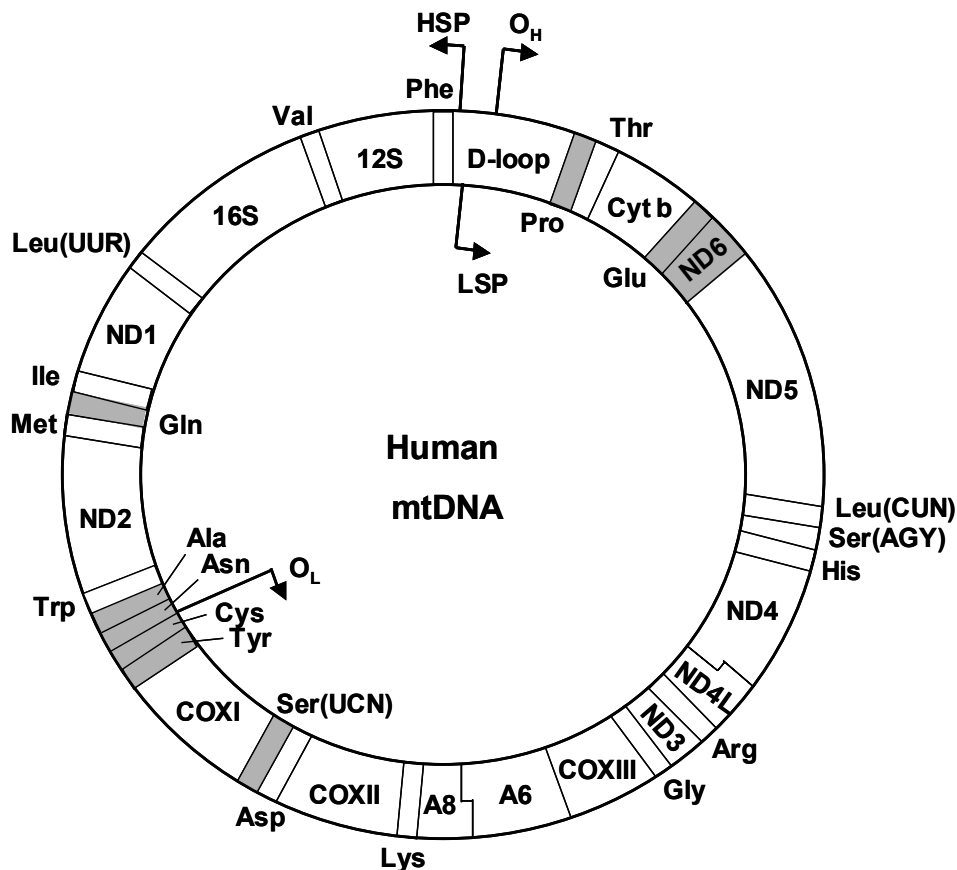
Figure 2.2. *The human mitochondrial genome. The 16.6 kb mtDNA encodes 13 polypeptides, 2 rRNAs (12S and 16S) and 22 tRNAs. The light-strand genes (8 tRNAs and ND6) are shown in grey. ND, NADH dehydrogenase; COX, cytochrome c oxidase; A, ATP synthase; Cyt b, cytochrome b; D-loop, displacement loop; O_H and O_L, origins of heavy- and light-strand replication; HSP and LSP, heavy- and light-strand promoters.*

2.1.4 Mitochondrial transcription, RNA processing and translation

MtDNA strands are transcribed from single promoters in their entirety as large polycistronic precursors (Montoya et al, 1982). Most of the RNA products are generated from the H-strand, including 10 mRNAs (encoding 12 of the polypeptides), 2 rRNAs and 14 tRNAs. There are two overlapping reading

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frames: for ND4 and ND4L mRNA, and for A6 and A8 mRNA. The remaining mRNA (ND6) and 8 tRNAs are transcribed from the L-strand.

For H-strand transcription, there is a second proposed promoter for transcription of the rDNA region that includes two rRNAs and tRNAs^{Val} and ^{Phe} (Montoya et al, 1982). This transcription unit is proposed to be responsible for the high rate of synthesis of rRNA (Montoya et al, 1983). Alternatively, the high steady-state levels of rRNA relative to mRNA could be regulated by selective termination of transcription from a single promoter (Clayton, 1992). The mitochondrial transcription termination factor (mTERF), with a binding site within the tRNA$^{Leu(UUR)}$ gene, has been proposed to play a role in the control of termination (Christianson and Clayton, 1988; Kruse et al, 1989).

The transcription machinery includes mitochondrial RNA polymerase (POLRMT), TFAM and either transcription factor B1 (TFB1M) or B2 (TFB2M) (see Gaspari et al, 2004). The primary polycistronic transcripts undergo endonucleolytic cleavage to release individual RNA molecules. According to the punctuation model (Ojala et al, 1981), tRNAs are interspersed between mRNAs and rRNAs and serve as signals for 5′- and 3′-processing enzymes that release also the flanking RNAs.

In human mitochondria, mRNAs and rRNAs are polyadenylated (Ojala et al, 1981). Polyadenylation is suggested to create the missing stop codons of some mRNAs (Ojala et al, 1981), which might be important for mRNA stability (Temperley et al, 2003). However, the role of polyadenylation in mRNA turnover remains controversial (Tomecki et al, 2004; Nagaike et al, 2005).

The 13 mRNA coding sequences are translated on mitochondrial ribosomes assembled from the 2 rRNAs and a dedicated set of ribosomal proteins that are encoded by the nuclear genome and imported into the mitochondria. Seventy-eight human mitochondrial ribosome proteins have been identified (Sylvester et al, 2004). In addition, the mitochondrial translation machinery requires several other nuclear-encoded components such as initiation, elongation and termination factors (see Towpik, 2005) and the enzymes for tRNA modification and aminoacylation.

2.2 Transfer RNAs

2.2.1 tRNA structure and function

Transfer RNA (tRNA) serves as an adaptor molecule that links the genetic code to translation. The main function of tRNA is to accept a specific amino acid and to deliver it to the ribosome. The enzymes that attach the amino acid to tRNA are called aminoacyl-tRNA synthetases (aaRSs). Multiple tRNAs recognized by a single enzyme are called isoacceptors.

Besides the key role in translation, both tRNAs and aaRSs are known to participate in other cellular processes. For example, tRNAs are involved in control of cognate aaRS expression by several regulatory mechanisms (reviewed by Ryckelynck et al, 2005). Specific tRNAs are used as primers for reverse transcription during replication of retroviruses and retrotransposons (reviewed by Mak and Kleiman, 1997). Thus, tRNA^{Lys,3} is packed into human HIV virus particles with assistance of LysRS, where it serves as a primer in replication cycle (reviewed by Kleiman and Cen, 2004). The essential functions of aaRSs include regulation of their own expression, tRNA transport, RNA splicing, amino acid biosynthesis and apoptosis (reviewed by Martinis et al, 1999; Francklyn et al, 2002). Mammalian aaRSs form a macromolecular complex with interacting multifunctional proteins, providing them novel roles in cell proliferation and immune response (reviewed by Park et al, 2005).

The secondary structure of tRNA forms a cloverleaf with four major arms named for their structure and function (see Goldman, 2005). The acceptor arm is terminated by the CCA sequence at the 3′-end to which the amino acid is attached. The anticodon arm contains the anticodon triplet in its loop. The TψC-arm (or T-arm) and D-arm are named for the presence of specific base modifications. Between the T- and the anticodon arm there is an extra arm, sometimes called the variable arm due to the high variability in its size in different tRNAs.

The tertiary structure of tRNA is L-shaped and consists of two domains (see Grosjean et al, 1996; Steinberg et al, 1997). The first domain is a 12 bp helix formed by the acceptor and T-stems, closed by the T-loop. The second domain is a biloop minihelix formed by the anticodon- and D-arms. These two domains are oriented at right angles and stabilized by tertiary interactions within the 3D core formed by the D- and T-loops.

tRNA contains many modified bases added posttranscriptionally by specific tRNA modification enzymes (Björk, 1995; Motorin and Grosjean, 2005). Base modifications play an important role in tRNA structural stabilization and functional properties, such as aminoacylation and codon binding (Björk, 1995; Agris 2004; Helm 2006).

2.2.2 Biogenesis of eukaryotic and prokaryotic tRNAs

Prokaryotic tRNA genes form clusters that are transcribed into long precursors. In mitochondria and chloroplasts, tRNA genes are scattered around the genome between mRNA and rRNA genes. Eukaryotic nuclear-coded tRNAs are transcribed by RNA polymerase III as precursors with 5′- and 3′-extensions. All tRNAs must be released from the primary transcripts by processing enzymes and further modified to become functional.

5´-end processing

RNase P is the major enzyme for endonucleolytic cleavage at the 5´-end that is ubiquitously present in bacteria, eukaryotes, mitochondria and chloroplasts (reviewed by Altman et al, 1995). Most forms of RNase P are ribonucleoproteins that consist of RNA subunit and protein subunits. These subunits cooperate to bind the substrate, with RNA catalytic subunit interacting with the tRNA domain and the protein subunit directly contributing to the binding affinity by contacting the 5´-leader sequence (Christian et al, 2002). tRNA recognition by RNase P is based on the conserved structure of coaxially stacked acceptor stem and T-stem and loop (Kurz and Fierke, 2000). The bacterial enzyme contacts also the 3´-end of tRNA, creating an essential '73-294'-interaction between the discriminator base of tRNA and the conserved U294 in M1 RNA subunit (Kirsebom, 2002) (Fig. 6.1b).

The RNase P RNA component from different organisms contains some conserved elements that are functionally essential (see Hsieh et al, 2004). Only the bacterial RNase P RNA is catalytically active in the absence of protein, although the protein subunit facilitates substrate recognition (Hsieh et al, 2004). The protein component of eukaryotic RNase P is much more complex than that of the bacterial enzyme, which could probably help to discriminate among potential RNA substrates (Xiao et al, 2002). In *E. coli*, RNase P is composed of a single RNA (M1) and a single protein (C5) subunit (Altman et al, 1995), whereas human RNase P, for example, has at least 10 different protein subunits associated with a single RNA subunit (H1) (Jarrous, 2002). The functions of individual subunits of eukaryotic RNase P may be also involved in interactions with other proteins that could play a role in maturation and assembly of the enzyme, as well as in regulation of distinct enzymatic activities (for example, RNA modification, mRNA processing) (Xiao et al, 2002; Jarrous, 2002). Mitochondrial tRNA processing enzymes are reviewed in section 2.3.5.

3´-end processing

Processing at the 3´-end of tRNAs is more variable and less well understood. In *E. coli*, removal of tRNA 3´-trailer includes several steps performed by a set of endo- and exonucleases (reviewed by Mörl and Marchfelder, 2001; Deutscher, 1995). It is initiated by an endonucleolytic cleavage downstream of the encoded CCA terminus, followed by an exonucleolytic trimming of some nucleotides at the 3´-end. The mature 3´-end is generated by a second exonucleolytic cleavage that occurs after processing of the 5´-end by RNase P (Deutscher, 1995). In eukaryotes, tRNA processing is more complex and, besides 5´-and 3´-cleavage, includes CCA sequence addition and sometimes intron removal (Deutscher, 1995). There seems to be no single pathway or definite order of processing for eukaryotic tRNAs. The majority of enzymes involved in 3´-processing are endonucleases, but exonucleases can act as a back-up system (see Mörl and

Marchfelder, 2001). In mitochondria and chloroplasts, processing at the 3′-end resembles the eukaryotic pathway and includes an endonucleolytic cleavage close to or at the discriminator nucleotide (Martin, 1995; Mörl and Marchfelder, 2001).

tRNase Z is an enzyme responsible for endonucleolytic cleavage at the 3′-end of tRNAs in bacteria, archaea and eukaryotes (see Schiffer et al, 2002). It belongs to the β-lactamase family of zinc-dependent metallohydrolases (de la Sierra-Gallay et al, 2005). Based on the crystal structure analysis of *Bacillus subtilis* tRNase Z, it forms a homodimer composed of a substrate recognition subunit with a long flexible arm and a catalytic subunit that performs cleavage. According to this model, the tRNA accommodation channel can tolerate only short 5′-extensions that could explain inhibition of tRNase Z by long 5′-leaders as observed previously (Nashimoto et al, 1999). Thus, the structural features of tRNase Z may contribute to the order of processing, which usually occurs at the 5′-end first. However, there are other factors that can modulate the order of processing.

CCA-addition

The presence of a 3′-CCA terminus is essential for tRNA aminoacylation, and the post-transcriptional addition of this sequence in most organisms, as well as repair of the encoded CCA in prokaryotes, is thus an important step of tRNA processing. The CCA sequence has also been demonstrated to be an antideterminant for eukaryotic tRNase Z which, in combination with tRNA localization, aminoacylation and transport could serve to prevent cycling of mature tRNA and inhibition of tRNase Z by the end product (Mohan et al, 1999). The antideterminant effect of the CCA sequence could be explained by a feature of tRNA Z structure that would result in a clash with the first C, which is facilitated by the second C (de la Sierra-Gallay et al, 2005).

CCA-adding enzymes (tRNA nucleotidyltransferases) belong to the nucleotidyltransferase superfamily, which can be divided into two classes (Yue et al, 1996). The archaeal enzyme (class I) and the eubacterial plus eukaryotic enzymes (class II) catalyse the same reaction but structurally are fundamentally different, suggesting that they have evolved independently (see Nakanishi and Nureki, 2005). On the other hand, bacterial tRNA nucleotidyltransferases and poly(A) polymerases, which catalyse different reactions, are highly similar in the catalytical core sequence, both belonging to the class II nucleotidyltransferase family (Betat et al, 2004). This suggests that the two enzymes were possibly interconverted during evolution (Yue et al, 1996; Betat et al, 2004).

tRNA nucleotidyltransferase is one of only three enzymes currently known to add nucleotides in a primer-dependent but template-independent mode (the other two being poly(A) polymerase and terminal deoxynucleotidyltransferase) (see Schürer et al, 2001). Several models are suggested for the mechanism of CCA addition, but it still remains debatable. The class I archaeal enzyme, for

example, is suggested to have a single active-site pocket for both C and A addition (Yue et al, 1998) that effects catalysis by a mechanism of progressive refolding of the growing 3´-terminus with the tRNA substrate fixed on the enzyme (Shi et al, 1998). In contrast, two distinct binding sites for ATP and CTP are proposed for the *E. coli* enzyme of class II that has a higher affinity for ATP and terminates polymerization after AMP incorporation (Tomari et al, 2000). A crystal structure of a class I enzyme supports the presence of a single nucleotide-binding pocket that changes its size and shape during progression of polymerization, which determines its specificity for CTP, ATP or termination (Xiong and Steitz, 2004).

Posttranscriptional modifications

About 100 different base modifications in tRNAs are known (Helm et al, 2006). Their functions can be divided into two categories: modifications in the tRNA core region contribute to tertiary structure stabilization; modifications within the anticodon loop are involved in codon recognition and aminoacylation (Nakanishi and Nureki, 2005). Depending on their sensitivity to tRNA structure, the modification enzymes can also be divided into two groups: group I enzymes require only local structural elements, whereas group II enzymes recognize overall 3D tRNA structure (Grosjean et al, 1996).

Modifications of both eukaryotic and bacterial tRNAs occur stepwise at certain stages of tRNA biosynthesis (Björk, 1995). According to the reinforcement hypothesis, group I modifications acting in the acceptor domain stabilize the T-loop and reinforce tertiary interactions with the D-loop that facilitate subsequent modification in the (initially loosely structured) anticodon domain (Helm, 2006).

The diversity and frequency of non-essential tRNA modifications (which constitute the majority) is not well understood. They could provide additional options for selective molecular recognition or contribute to unique structural folds of tRNA (Engelke and Hopper, 2006). Recently, a novel rapid tRNA degradation (RTD) pathway that eliminates undermodified mature tRNA in yeast was reported, suggesting a critical role of non-essential modifications for tRNA stability (Alexandrov et al, 2006).

2.2.3 The ribosome cycle

The ribosome cycle consists of three major steps: binding of aa-tRNA, transpeptidation and translocation. The ribosome contains three binding sites for tRNA: the A-site for aa-tRNA (decoding center), the P-site for peptidyl-tRNA, and the E- (exit) site for deacylated tRNA. Aa-tRNA is delivered to the ribosome in a ternary complex with elongation factor EF-Tu (eEF1A in eukaryotes), bound to GTP. Accommodation of tRNA in the A-site is accomplished by GTP

hydrolysis and release of EF-Tu:GDP. EF-Tu is reactivated by exchange of GDP for GTP through an intermediate complex with EF-Ts (eEF1B in eukaryotes). At the next stage, a peptidyl transfer from the P-site peptidyl-tRNA to the A-site aa-tRNA occurs. The elongated peptidyl-tRNA is translocated from the A-site to the P-site, and the deacylated tRNA moves to the E-site. This step is catalysed by EF-G:GTP (Rodnina et al, 1997).

GTP-dependent elongation factors act as catalysts that decrease the high kinetic barriers for conformational transition states of the translation cycle, although the factor-free translation is also possible (see review by Spirin, 2002). According to the locking-unlocking concept applied to the ribosome, the requirement for substrate movement, on one hand, and its tight fixation, on the other, is achieved by oscillation of the macromolecular complex between open, closed and intermediate states (Spirin, 2002).

GTP hydrolysis on EF-Tu separates two steps in the selection of cognate aa-tRNA, initial selection and proofreading, before its accommodation in the A-site of the ribosome (Rodnina and Wintermeyer, 2001). The conformational changes in tRNA are essential for its own acceptance and the fidelity of translation (Cochella and Green, 2005). A kink between the D- and the anticodon arms acts as a molecular spring swinging tRNA to the A-site after GTP hydrolysis (Valle et al, 2003). The energy cost of tRNA deformation is compensated by a codon-anticodon pairing that is more stable in the cognate complex and lead to rapid hydrolysis of GTP, whereas lack of tight interactions would result in rejection of aa-tRNA.

The basic steps of translation are conserved in mammalian mitochondria (Woriax et al, 1997). Mammalian and *S. cerevisiae* mitochondrial EF-Tu are highly similar in sequence and functionally analogous to the prokaryotic factor (Rosenthal and Bodley, 1987; Woriax et al, 1995; Hunter and Spremulli, 2004). Both factors, however, differ significantly from *E. coli* EF-Tu in having a lower affinity to guanine nucleotides (Rosenthal and Bodley, 1987; Cai et al, 2000). *S. cerevisiae* mitochondrial EF-Tu does not require the exchange factor EF-Ts that is found in mitochondria of human and *S. pombe* (Chiron et al, 2005).

2. 3 Mitochondrial tRNAs

2.3.1 Structural features

Many mitochondrial tRNAs in general have unusual features of structure, lack of conserved bases found in other tRNAs and different rules for secondary and tertiary interactions (Dirheimer, 1995). For mammalian mitochondrial tRNAs these features may be summarized as follows (Helm et al, 2000):

- The primary structure of mitochondrial tRNAs is not well conserved, but the degree of conservation varies within tRNA families. D- and T-loops are poor

in G content in tRNAs transcribed from the heavy DNA strand ('light' tRNAs) and poor in C content in tRNAs transcribed from the light strand ('heavy' tRNAs).

   - With the exception of two seryl-tRNAs, other 20 tRNAs fold into classical cloverleaves. $tRNA^{Ser(AGY)}$ is missing the D-arm, and $tRNA^{Ser(UCN)}$ forms an unusual cloverleaf (see section 2.3.2). The size of D- and T-loops can vary to large extents with a tendency towards small loops. The stem regions are rich in mismatches in 'light' tRNAs and rich in G-U pairs in 'heavy' tRNAs. In combination with a decreased GC content in stem regions, this leads to thermodynamic instability of mitochondrial tRNAs that may increase their sensitivity to pathogenic mutations (Wittenhagen and Kelley, 2003).

   - Most mitochondrial tRNAs have only a few classical tertiary interactions that are possible only within the core, excluding D- and T-loop interactions. Mitochondrial tRNAs are able to fold into the conventional L-shape, but it is based on weaker tertiary structures or alternative interactions. For $tRNA^{Ser(AGY)}$, a boomerang model is proposed (Steinberg et al, 1994).

2.3.2 tRNA$^{Ser(UCN)}$

In mammalian mitochondrial genomes, there are two serine isoacceptor tRNAs specific for AGY and UCN codons, respectively. Both tRNAs have unusual secondary structures, $tRNA^{Ser(AGY)}$ lacking the entire D-arm and $tRNA^{Ser(UCN)}$ folding into a slightly altered cloverleaf. The novel cloverleaf structure with only one nucleotide (usually 2) in the connector between the acceptor and D-stem is conserved only in mammalian mitochondrial $tRNA^{Ser(UCN)}$ genes (Yokogawa et al, 1991). In such a structure, there are 7 base pairs in the acceptor stem, 4 base pairs in the D-stem and 6 base pairs (usually 5) in the anticodon stem (Yokogawa et al, 1991). The secondary structure of $tRNA^{Ser(UCN)}$, however, is rather weak (Yokogawa et al, 1989), probably due to the presence in total of 5 G-U base pairs. Although there are missing core interactions in $tRNA^{Ser(UCN)}$, it is one of only a few mitochondrial tRNAs that have the conventional GG and UUCG sequences in their D- and T-loops, respectively, which allow canonical tertiary interactions between these loops (Watanabe et al, 1994; Helm et al, 2000). These interactions preserve the L-shaped higher-order structure with an inverse relationship between elongated anticodon stem length and the number of D-domain nucleotide layers to keep the distance and orientation between the anticodon and CCA-end region constant (Watanabe et al, 1994; Steinberg et al, 1997).

Both serine isoacceptor tRNAs were demonstrated to be functional in translation *in vitro*, although $tRNA^{Ser(AGY)}$ had a lower translational ability (Hanada et al, 2001). Both tRNAs could form strong ternary complexes with *E. coli* and mitochondrial EF-Tu *in vitro* (Hunter and Spremulli, 2004). Interestingly, a distinct EF-Tu exists for seryl-tRNAs lacking the D-arm in nematode mitochondria (Ohtsuki et al, 2002).

2.3.3 Mitochondrial seryl-tRNA synthetase (mt SerRS)

Although two isoacceptor tRNAs have no common structure, they are recognized by a single mitochondrial seryl-tRNA synthetase (mt SerRS) (Shimada et al, 2001). As an exception from other organisms and organelles, animal mitochondrial tRNA^{Ser} isoacceptors do not carry a long extra arm that serves as a specific recognition element for seryl-tRNA synthetases (Lenhard et al, 1999). Instead, mt SerRS recognizes the T-loop of both tRNAs (Shimada et al, 2001). The mode of recognition of the two serine tRNAs is distinct, as tertiary interactions between the T- and D-loops are required for recognition of tRNA$^{Ser(UCN)}$ (Shimada et al, 2001). The N-terminal region of mt SerRS involved in tRNA binding has a low degree of similarity with other SerRS, whereas the catalytic core in the C-terminal region is highly conserved (Yokogawa et al, 2000).

Recently, a dual-mode recognition mechanism was suggested based on the crystal structure of mammalian mt SerRS (Chimnaronk et al, 2005). The unique extensions at both N- and C-termini, the distal helix and the C-tail, respectively, and a narrow positively charged patch on the N-terminal helical arm of the enzyme are essential for tRNA binding. These three regions of mt SerRS act synergistically to lock the T-loop of tRNA, the distal helix and C-tail contacting the major groove side of the acceptor helix of tRNA, and the positively charged patch on the helical arm contacting the opposite side of the acceptor helix. A dual-mode recognition involves a distinct set of amino acid residues for each isoacceptor and an alternative combination of the recognition sites of mt SerRS.

2.3.4 Aminoacylation

Mitochondrial and cytosolic aminoacyl-tRNA synthetases (aaRSs) are encoded by different sets of nuclear genes, with the exception of LysRS and GlyRS (Florentz et al, 2003; Bonnefond et al, 2005a). Two forms of GlyRS are translated from different initiation codons, one of which supplies an N-terminal mitochondrial targeting sequence (Shiba et al, 1994). LysRS isoforms are created by alternative splicing with the mitochondrial targeting sequence encoded on a dedicated exon (Tolkunova et al, 2000).

As generalized from studies on the classical bacterial tRNA model, the major sites of tRNA recognition by cognate aaRS are located in the anticodon, in the distal part of the acceptor arm, or in the variable arm (reviewed by McClain, 1993). The discriminator base (N73) contributes to the identity of virtually every *E. coli* tRNA species. Moreover, substrate recognition depends not only on sequence-specific interactions, but also on the ability of tRNA to undergo specific conformational changes.

Aminoacylation identity elements of mammalian mitochondrial tRNAs are not well understood. Some tRNAs, for example tRNA^{Tyr} and tRNA^{Asn}, possess all elements necessary for aminoacylation by *E. coli* enzymes, whereas in most

tRNAs only some conserved elements are present (Helm et al, 2000). Among them, the discriminator base (N73) is the most conserved. The aminoacylation rules in mitochondria may be different due to unusual structural features of mitochondrial tRNAs and mitochondrial aaRSs (Florentz et al, 2003). For instance, the human mitochondrial TyrRS uses unusual recognition rules, having lost the identity elements in the acceptor branch of tRNA^{Tyr} (Bonnefond et al, 2005b). Perhaps, the most unique example is mt SerRS, which uses different mechanisms to recognize each of its isoacceptor tRNAs (see section 2.3.3).

For human mitochondrial tRNA^{Lys}, the m^{1}A9 modification is essential for both folding (Helm et al, 1998) and aminoacylation, and the major lysylation element is the central residue in the anticodon loop (U35) (Sissler et al, 2004).

Although the transcript of human mitochondrial tRNA$^{Leu(UUR)}$ folds only partially into the conventional L-shape structure with loose D- and anticodon arms forming several alternative conformations, it can nevertheless be aminoacylated (Sohm et al, 2003). The identity elements for human mitochondrial LeuRS are located in the acceptor stem, the anticodon stem and the D-loop of tRNA$^{Leu(UUR)}$, representing a new pattern of tRNA recognition, the discriminator base being the only conserved element for leucylation in other systems (Sohm et al, 2004). The efficiency of aminoacylation for this structurally comparatively unstable tRNA is dependent on the stability of the anticodon stem (Zagryadskaya and Kelley, 2005). Binding by LeuRS induces conformational changes stabilizing the helical domains of the tRNA (Sohm et al, 2004).

The unique feature of the second leucine isoacceptor in human mitochondria, tRNA$^{Leu(CUN)}$, is its flexibility due to a low number of GC pairs in the T-stem, which enables a 1 nt slip (Hao et al, 2005). Based on *in vitro* aminoacylation analysis, two resulting conformations are proposed to function as a switch for the charging capacity of human mitochondrial tRNA$^{Leu(CUN)}$ via a self-regulation mechanism (Hao et al, 2005). While the exact identity elements for recognition of the leucine tRNAs are not known, this might indicate that the two leucine isoacceptors are also recognized by different mechanisms.

From an evolutionary point of view, one striking feature of at least some mitochondrial aaRSs could be a tendency towards a loss of editing activity for misacylated tRNAs (Roy H et al, 2005). Other factors compensating for the loss of this activity are not known, but the quality of mitochondrial protein synthesis might be controlled by another mechanism, such as efficient post-translational protein degradation (Roy H et al, 2005).

2.3.5 Mitochondrial tRNA processing enzymes

RNase P

Mitochondrial RNase P enzymes are diverse in architecture. Some primitive eukaryotes contain a bacterial-like gene for the RNA subunit, whereas in

Trypanosoma mitochondria, as well as in plant chloroplasts, RNase P is composed solely of protein (Gopalan et al, 2001). In plant chloroplasts, the catalytic mechanism of RNase P is different, and the enzyme is suggested to be evolutionarily unrelated to the others (see Gopalan et al, 2001). In *S. cerevisiae* mitochondria, the RNA subunit is encoded by the mitochondrial genome and the protein subunit is nuclear-encoded (see Xiao et al, 2002).

In human, some protein subunits of RNase P show homology with corresponding yeast proteins, and several Rpp subunits are conserved in archaea (see Jarrous, 2002). The nuclear and the mitochondrial forms of human RNase P have been shown to contain an identical H1 RNA subunit (Puranam and Attardi, 2001). Controversially, previous studies suggested that human mitochondrial RNase P is not a ribonucleoprotein but a protein enzyme (Rossmanith and Karwan, 1998). The possible presence of H1 RNA in the mitochondrial enzyme raises a question about its import mechanism into mitochondria (Jacobs, 2001). Such a mechanism exists for nuclear-encoded tRNA import to mitochondria in several organisms, as well as for 5S rRNA and RNase MRP in mammalian cells (Chang and Clayton, 1987b; Schneider and Marechal-Drouard, 2000; Entelis et al, 2001). Only a small proportion of H1 RNA is shown to be associated with mitochondria, probably implying that import of RNase P is a highly regulated process, and that RNase P may play a regulatory role in mitochondrial tRNA synthesis (Puranam and Attardi, 2001). The activity of human nuclear RNase P can be modulated by the La protein, which binds to the tRNA precursor and blocks the 5´-processing site (Intine et al, 2000), and by alternative splicing of the Rpp21 subunit that generates a variant not associated with catalytically active RNase P (Jarrous et al, 2001).

Another outstanding question concerns whether RNase P enters mitochondria as an intact enzyme or is assembled from its components inside mitochondria. In the human cell nucleus, the distribution of H1 RNA and RNase P protein subunits is different, suggesting a dynamic assembly of RNase P at sites of active gene transcription (see Jarrous, 2002).

tRNase Z

tRNase Z belongs to the conserved ELAC1/ELAC2 group of proteins (Tavtigian et al, 2001). *ELAC1* genes encode a short form of the enzyme that is present in all kingdoms of organisms, whereas longer ELAC2 proteins that appear to have evolved from duplication of ELAC1 proteins are exclusively found in eukaryotes (Schiffer at al, 2002; Dubrovsky et al, 2004). In *Drosophila, C. elegans* and *S. cerevisiae* only the long form of tRNase Z is encoded, whereas genomes of human and *A. thaliana* contain both short and long forms (Takaku et al, 2004). These two forms of tRNase Z may represent the nuclear and the mitochondrial processing enzymes. For human ELAC2, a mitochondrial localization is predicted, although the enzyme can be detected both in cytoplasm and nuclei (Takaku, 2003). The long form of the enzyme, however, has been

demonstrated to be much more efficient in the processing of a human nuclear-encoded tRNA *in vitro* than the short form (Yan H et al, 2006). On the other hand, the long forms of tRNaze Z may have additional roles in RNA metabolism. For example, in *Drosophila*, the Jhl-1 gene that encodes the ELAC2 protein is proposed to be essential also for the translational capacity of the developing embryo (Dubrovsky et al, 2004). In humans, ELAC2 was first identified as a candidate prostate cancer susceptibility gene (Tavtigian et al, 2001). In support of this, the two forms of tRNase Z have been demonstrated to have different substrate specificities due to the N-terminal part of the ELAC2 protein that is not essential for tRNA processing activity (Takaku et al, 2004).

tRNA nucleotidyltransferase

Mitochondrial tRNAs generally do not encode the CCA terminus (Martin, 1995). In yeast, cytoplasmic and mitochondrial CCA-adding enzymes are encoded by a single gene, *CCA1*, which has three ATG codons at the 5′-end of its ORF (Chen et al, 1992). The polypeptide translated from the first ATG is localized primarily to mitochondria, whereas polypeptides translated starting at the second and third ATG codons are found mainly in the cytosol and also in the nucleus (Wolfe et al, 1996).

In human and mouse, a single gene with only one ATG was found to encode CCA-adding activity (Nagaike et al, 2001). The human recombinant enzyme was able to repair both mitochondrial and yeast cytoplasmic tRNAs with high efficiency that suggests a loose recognition of non-conserved tRNA structure (Nagaike et al, 2001).

2.3.6 Mitochondrial tRNA modification enzymes

In mitochondria, posttranscriptional modifications may play some specific roles, such as facilitation of more extensive wobble codon recognition due to fewer tRNAs than encoded in the nucleus, or determination for mitochondrial import of cytoplasmic tRNAs (Agris, 2004). As a rule, modifications of mitochondrial tRNAs are less abundant and less variable than those in cytoplasmic tRNAs (Motorin and Grosjean, 2005). Some modifications may compensate for loss of secondary and tertiary structure by mitochondrial tRNAs (Helm et al, 2004). For example, m^1A9, a modification unique to animal mitochondria, is required for correct folding of human mitochondrial tRNA^{Lys} (Helm et al, 2004). *In vitro* transcribed human mitochondrial tRNA$^{Leu(UUR)}$ only partially folds into an L-shaped structure with a floppy anticodon branch (Sohm et al, 2003).

Generally, mammalian mitochondrial tRNA modifications and their synthesis are not well characterized. Some of the mitochondrial modification enzymes may resemble their prokaryotic equivalents: for example, *E. coli* methylguanosine

transferase (1MGT) methylated a human mitochondrial tRNA transcript *in vitro* at the same efficiency as its natural substrate (Brule et al, 1998). In yeast, there are few examples of single genes encoding both nuclear/cytoplasmic and mitochondrial enzymes, as well as of distinct nuclear genes encoding compartment-specialized isozymes with high degree of homology (Motorin and Grosjean, 2005). The distribution of human pseudouridine synthase (Pus1p) was shown to be nuclear, cytoplasmic and mitochondrial (Patton et al, 2005), implying that common enzymes may perform some ubiquitous tRNA modifications.

Some specific mitochondrial tRNA modification enzymes were identified in connection with mitochondrial pathologies. MTU1 was characterized as a mitochondrial tRNA-specific 2-thiouridylase responsible for wobble modification in human and yeast mitochondrial tRNAs (Umeda et al, 2005). Novel taurine-containing uridine modifications were lacking in mitochondrial tRNAs bearing pathological mutations (see section on mitochondrial tRNA mutations). The biosynthesis of these modifications requires many genes, some of which were identified previously as nuclear modifiers of phenotypic expression of pathological mtDNA mutations. In yeast, Mto1p and Mss1p have been shown to be involved in the initial steps of this biosynthetic pathway (Umeda et al, 2005). The human homologs are MTO1 (Li X et al, 2002) and GTPBP3 (Li and Guan, 2002), respectively. Recently, MTO2 (TRMU) was proposed to be another member of the same pathway in yeast and humans (Yan et al, 2005; Yan Q et al, 2006).

2.4 Mitochondrial disorders

2.4.1 Overview

Mitochondrial diseases can be divided into two major groups either caused by mutations in ncDNA or in mtDNA.

The major groups of mitochondrial disease-causing mutations in ncDNA (see reviews by Zeviani and Klopstock 2001; DiMauro and Gurgel-Giannetti, 2005) include those in subunits of the respiratory chain complexes, mutations in proteins required for the assembly and function of the respiratory chain complexes, mutations influencing biogenesis of mitochondria, and mutations in genes involved in the control of abundance and stability of mtDNA. With further understanding of the involvement of nuclear genes in mitochondrial transcription and translation, the connection to mitochonrial diseases is expanding to factors acting at the level of tRNA modification, regulation of translation and transcription-coupled regulation of mitochondrial RNA metabolism (Shadel, 2004; Jacobs and Turnbull, 2005).

MtDNA is maternally inherited (Giles et al, 1980). Whereas multiple copies of mtDNA are mostly identical in unaffected individuals, in patients, the proportion of mutant and wild-type mtDNA may vary within cells and tissues, a situation described as heteroplasmy. The transmission of heteroplasmy is suggested to occur by a combination of the genetic bottleneck and possible preferential segregation of mutant mtDNA (Poulton et al, 1998). For some mutations, a selective proliferation may account for a positive selection of mutant mtDNA in postmitotic tissues (Chinnery et al, 2000). Heteroplasmy is deleterious only after exceeding a critical level, i.e. a threshold that may vary for different mutations and in different cell types.

MtDNA defects are divided into two groups: large rearrangements and point mutations (Fernandez-Moreno et al, 2000; Schapira, 2002; Schmiedel et al, 2003). MtDNA rearrangements are usually sporadic and include a single large deletion, small-scale deletions or, more rarely, duplications. Large-scale deletions were the first identified mtDNA mutations (Holt et al, 1988). They include progressive external ophthalmoplegia (PEO), Kearns-Sayre syndrome (KSS) and Pearson syndrome. In some patients, mtDNA deletions were found to co-exist with duplicated molecules that could represent an intermediate form of deletions (Poulton et al, 1993).

Point mutations may affect any gene of mtDNA. They are usually maternally inherited and affect the nervous system and skeletal muscle. The first disease-causing mutation found in a mitochondrial protein-coding gene, the G11778A mutation in ND4, is associated with Leber hereditary optic neuropathy (LHON) (Wallace et al, 1988). The specific features of this syndrome include restricted organ specificity affecting the retinal ganglion cells and the optic nerve, sex bias to males, and often homoplasmic levels of the mutations. Two other frequent LHON-causing mutations are G3460A in ND1 and T14484C in ND6. The mechanism of the expression of this disease is not understood, but mtDNA haplogroup-association, nuclear modifier and environmental factors may play a role in its penetrance (Man et al, 2002; Chinnery and Schon, 2003). A recently identified X-chromosomal modifier locus has been proposed to play a role in LHON penetrance and sex bias (Hudson et al, 2005). The penetrance and expressivity of the G11778A mutation could be increased by mitochondrial tRNA point mutations presumably interfering with tRNA metabolism (Li et al, 2006; Qu et al, 2006). The clinical expression of the T8993G mutation in the A6 gene is dependent on the level of heteroplasmy and is associated either with NARP syndrome (neuropathy, ataxia and retinitis pigmentosa) or, at levels over 90%, with more severe Leigh syndrome (infantile subacute necrotizing encephalopathy).

The most frequent mutation in rRNA is the A1555G mutation in 12S rRNA associated with both aminoglycoside-induced and non-syndromic deafness. The features of this mutation are homoplasmy and variable penetrance in families with different ethnic backgrounds. In some families, nuclear modifiers may determine the biochemical phenotype of the mutation (Guan et al, 2001; Li and Guan, 2002; Li X et al, 2002), whereas in others, aminoglycoside exposure

seems to be a major interacting factor (Young et al, 2005). The common effect of such modifiers might be a decrease in the overall rate of mitochondrial translation, as suggested in the case of a mutation in the mitochondrial tRNA modification enzyme TRMU, which results in a defect of tRNA metabolism (Guan et al, 2006).

tRNA point mutations

tRNA point mutations (Fig. 2.3) are the cause of the largest and the most diverse group of mtDNA disorders. The first two identified tRNA mutations, A8344G in tRNA^{Lys} and A3243G in tRNA$^{Leu(UUR)}$, are amongst the most common and most studied mutations.

The A3243G mutation is associated with a broad variety of clinical phenotypes and is a common cause for MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) (Goto et al, 1990; Kobayashi et al, 1990). At low levels, the mutation may cause only type II diabetes in combination with deafness (van den Ouweland et al, 1994). MELAS is a frequent result also of other mutations in tRNA $^{Leu(UUR)}$.

The A8344G mutation is also associated with a mitochondrial encephalopathy, known as MERRF syndrome (myoclonic epilepsy and ragged-red fibers) (Shoffner et al, 1990) that is represented by several other mutations in tRNA^{Lys} as well.

Another well-established correlation with clinical presentations is known for mutations in tRNA$^{Ser(UCN)}$ that are mainly deafness-associated (Reid et al, 1994; Tiranti et al, 1995) and for mutations in tRNA^{Ile} associated with cardiomyopathy (Taniike et al, 1992; Casali et al, 1995) or PEO (Silvestri et al, 1996; Taylor et al, 1998).

The pathological mechanisms of mitochondrial tRNA point mutations are poorly understood. Whereas the molecular effects of some mutations have been elucidated (see section 2.4.3), their correlation with disease phenotype as well as the penetrance pattern in different patients remains extremely complex. Such factors as levels of heteroplasmy, as well as more globally acting genetic and environmental factors, could modulate the expression of the disease. The OXPHOS status, in its turn, might affect nuclear gene expression that is suggested to contribute, for example, to association of the A3243G mutation with diabetes (Tafrechi et al, 2005).

Heteroplasmy

Heteroplasmy is considered to be one of the criteria of pathogenicity, indicating that a mutation has not been fixed but also is not a polymorphism (Chinnery and Schon, 2003). On the other hand, homoplasmic pathogenic mutations could remain asymptomatic in some patients, implying the importance

of additional factors. Such examples are known for the LHON mutations, the A1555G mutation, the A4300G mutation in tRNA[Ile] and the T14709C mutation in tRNA[Glu] (Carelli et al, 2003; DiMauro and Gurgel-Giannetti 2005).

The levels of heteroplasmy do not always well correlate with the severity and specificity of the clinical phenotype (Morgan-Hughes et al, 1995; Schon et al, 2000). Quantitatively, the same level of heteroplasmy may have different effects on mitochondrial function, depending on the tissue distribution of the mutant mtDNA. Information about the tissue distribution of the mutation is usually very restricted, due to the limited availability of biopsy material, but may vary greatly depending on the source of the sample even within same tissue.

For respiratory complexes, the threshold effect is higher assuming a model of homogeneous distribution of the deficiency in all mitochondria, compared to the model of complete deficiency in some mitochondria (Korzeniewski et al, 2001). If the same is true for ROS production, a clustering of mutant mitochondria may result in significant oxidative damage to the whole cell. Some mtDNA mutations, such as A3243G and A8344G, have been demonstrated to increase ROS production in cultured cells (Vives-Bauza et al, 2006). Since antioxidant enzyme activity was also increased, the resulting balance in ROS production may be one of the factors contributing to the variability of the clinical phenotypes. In another study, a correlation between the degree of heteroplasmy of the A8344G mutation and oxidative damage to patient cells was found, which was present even in asymptomatic patients with moderate heteroplasmy (Canter et al, 2005). In addition, variation in biochemical threshold is proposed to explain many aspects of tissue specificity (Rossignol et al, 1999).

MtDNA mutations may be unequally distributed also at the level of organs or the whole organism, due to the clonal expansion of stem cells during development (Khrapko et al, 2003). The mechanism of segregation of heteroplasmic mtDNA may not be entirely random. Mutant mtDNA has been suggested to have a replicative advantage over wild-type mtDNA (Yoneda et al, 1992). In cultured cells, a shift towards the mutant or wild type mtDNA was observed to be dependent also on the nuclear background (Dunbar et al, 1995). Studies in a mouse model suggest that mtDNA segregation is tissue-specific, as well as age-dependent (Battersby et al, 2003).
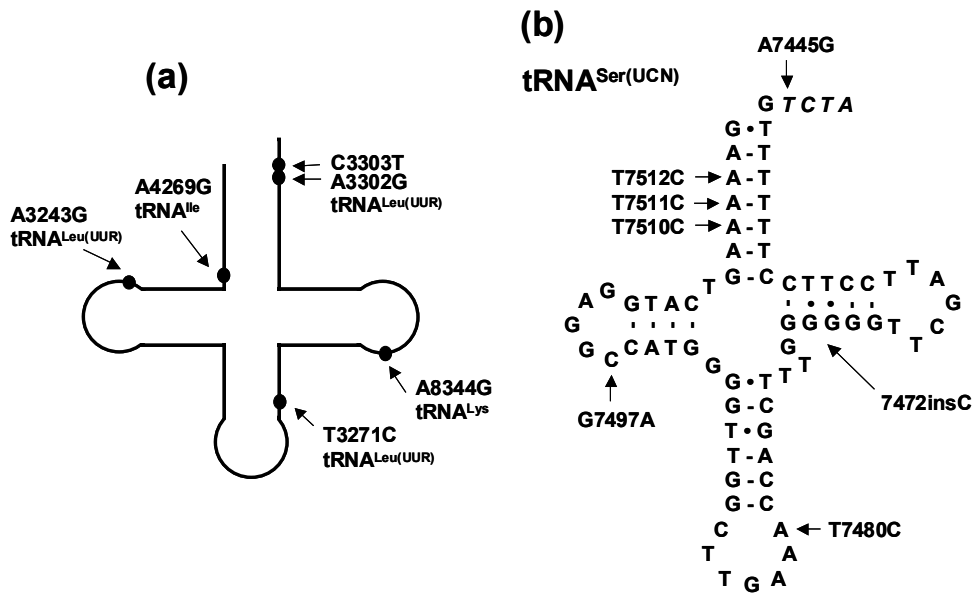
Figure 2.3. *Examples of pathogenic point mutations in human mitochondrial tRNA genes (a) and pathogenic mutations, mainly associated with sensorineural deafness, in tRNA^Ser(UCN) gene (b).*

2.4.2 Deafness-associated mutations

Hearing loss is a common consequence of mitochondrial dysfunction, although the tissue specificity of deafness-associated mutations remains unclear. Mitochondrial deafness can be part of systemic neuromuscular syndromes such as MELAS, MERRF and KS syndrome (Fischel-Ghodsian, 2003). Non-syndromic cases of sensorineural deafness are associated mainly with the A1555G mutation in 12S rRNA and with several point mutations in mitochondrial tRNAs, most frequently found in tRNA^Ser(UCN). Many of these mutations are homoplasmic or have a high threshold for the expression of the disease, implying a non-deleterious effect in most contexts or tissues. A specific involvement of cochlear cells may be partially explained by genetic and environmental modifiers or interference with tissue-specific components and products of mitochondrial translation (Jacobs, 1997; Fischel-Ghodsian, 2003).

tRNA^Ser(UCN) is a hot spot for mutations with sensorineural deafness as a primary pathological symptom, sometimes combined with more complex syndromic features (Fig. 2.3b). For example, the first described mutation in this tRNA, A7445G (Reid et al, 1994), lying 1 np beyond its 3′-end, is associated with deafness and palmoplantar keratoderma (Sevior et al, 1998). The 7472insC mutation in some patients causes a wider neurological syndrome, including deafness, ataxia and myoclonus to a variable combination and degree (Tiranti et al, 1995). Other deafness-associated mutations found in tRNA^Ser(UCN) include T7510C (Hutchin et al, 2000), T7511C (Sue et al, 1999), T7512C (Jaksch et al, 1998), T7480C (Bidooki et al, 2004) and G7497A (Jaksch et al, 1998; Möllers et

al, 2005). The G7444A mutation adjacent to the 3′-end of tRNA$^{Ser(UCN)}$ coexisting with the A1555G mutation, is proposed to act as a modifier for its phenotypic expression, either by abolishing the COXI stop codon or by interfering with tRNA processing (Pandya et al, 1999; Yuan et al, 2005).

At least one mutation in tRNA$^{Ser(AGY)}$, C12258A, is also associated with deafness in combination with diabetes (Lynn et al, 1998) or with Usher-like syndrome including sensorineural hearing loss and retinitis pigmentosa (Mansergh et al, 1999).

The human mt SerRS gene is located on chromosome 19q13.1 adjacent to the 5′-end of the *MRPS12* gene encoding the mitochondrial ribosomal protein S12 (Yokogawa et al, 2000; Shah et al, 1998). The two genes are transcribed from a common bidirectional promoter. Both genes might be considered as candidates for involvement in sensorineural deafness. Tissue-specific difference in the levels of mt SerRS expression observed in mouse (Gibbons et al, 2004) could be possibly one of the modifying factors for the disease phenotype.

The 7472insC mutation

The 7472insC mutation was first identified in a Sicilian family by Tiranti et al in 1995. The hearing impairment was clearly the most expressed, and in many cases the only neurological symptom, even in the individuals carrying nearly homoplasmic levels of the mutation. The wider neurological syndrome accompanying the deafness in some patients often developed with age. The mutation became pathogenic only at high levels of heteroplasmy, and individuals with up to 76% of mutant mtDNA remained unaffected. The most severe syndrome was present in patients with about 95% of heteroplasmy. At such a high level of heteroplasmy a combined deficiency of complex I and IV was detectable in the muscle tissue. Generally, there was a correlation between the level of heteroplasmy and the expression of disease, but it would not well explain all the variability of the clinical and biochemical phenotype.

The 7472insC mutation was found in several other families, also with different penetration of the disease. In a large Dutch family the mutation caused only hearing loss, with the exception of one patient who developed additional neurological symptoms (Verhoeven et al, 1999). This patient carried nearly homoplasmic levels (99%) of the mutation, whereas in most individuals the heteroplasmy level was over 90%. There was no significant correlation between the level of heteroplasmy and the degree of hearing impairment or age of onset, and generally the expression of the disease was milder than in the Sicilian family. (Note that the number of patients in the Dutch family (38) was significantly higher than in the Sicilian family (13), but the proportion of unaffected persons was ~30% in both cases.)

These two broad studies give an example of the variability of the disease caused by the 7472insC mutation not only between families, but also between individuals in the same family. On the other hand, a distinct syndrome of

progressive myoclonus epilepsy and sensorineural deafness was caused by two different mutations, 7472insC and T7512C, in different families (Jaksch et al, 1998). Several patients from five families had homoplasmic levels of the respective mutations. In one case with the 7472insC mutation the consequences of the disease were lethal, although the mutation causes generally a mild phenotype.

Although the mutation is usually expressed at a high threshold of heteroplasmy (over 85%), a much lower level of heteroplasmy has been found associated with sensorineural deafness: 70% in blood in one case, and 10% in blood, 42% in hair in another (Jacobs et al, 2005). A mutation load below this threshold has been reported to cause deafness also in another study (Fetoni et al, 2004).

2.4.3 Molecular effects of tRNA mutations

Mutations in mitochondrial tRNAs can be either pathogenic or neutral polymorphisms. Several attempts have been made to identify the criteria for pathogenic mutations, which can be summarized as location in evolutionary conserved sites, primarily in the stem structures with the disruption of Watson-Crick base pairing (Florentz and Sissler, 2001; McFarland et al, 2004; Kondrashov 2005). Moreover, a tendency for compensatory evolution restoring the base pairing in Great Apes (i.e. closely related species to human) could increase the accuracy of such analysis (Kondrashov, 2005). Although disease-related mutations are found in all but one mitochondrial tRNA (tRNA^{Arg} being the exception), they predominate in tRNA$^{Leu(UUR)}$, tRNA^{Lys}, and tRNA^{Ile} (see Florentz et al, 2003; Wittenhagen and Kelley, 2003). A weakly stabilized tertiary structure of tRNA$^{Leu(UUR)}$ (Sohm et al, 2003) and tRNA^{Lys} (Helm et al, 2004) and a high content of A-U base pairs in tRNA^{Ile} (Kelley et al, 2000) may be considered as a reason for their vulnerability to pathogenic mutations. In tRNA$^{Leu(UUR)}$, several mutations, including A3243G, are located in the D- and T-loops, which have an unusually large size and therefore might particularly rely on base composition in maintenance of tRNA structure (McFarland et al, 2004). The same argument could be applied to mutations occurring in large loops of other tRNAs, for example, the A8344G mutation in tRNA^{Lys}, the tRNA with the largest T-loop (McFarland et al, 2004).

The common consequence of tRNA point mutations is generally assumed to be an impaired mitochondrial translation due to a deficiency of functional tRNA. However, the phenotypic consequences of this correlation are more complex and could depend on codon specificity (see Jacobs, 2003) and a threshold value for individual tRNA. For example, in cybrid cells, the extremely low levels of tRNA$^{Ser(UCN)}$ due to the G7497A and T7512C mutations still maintained the efficiency of protein synthesis at about 60% of wild-type level (Möllers et al, 2005). Comparable levels of functional tRNA^{Lys} with the G8313A mutation in the same cellular background induced strong translational defects (Bacman et al,

2003). At the level of clinical presentation, this correlation is more variable and may depend on a number of factors.

At the molecular level, tRNA mutations may have a combination of different pathological effects, as in the case of the A3243G MELAS or A8344G MERRF mutations. The A3243G mutation, for example, is predicted to produce a pronounced defect in the tertiary structure of tRNA$^{Leu(UUR)}$ due to the loss of the stabilizing reverse Hoogsteen pairing between the affected nucleotide A14 and U8 (Wittenhagen and Kelley, 2003), and therefore may interfere with multiple steps of tRNA maturation and function. A unique feature of the A3243G mutation is the ability to form a dimeric complex through a self-complementary hexanucleotide sequence within the D-arm of tRNA$^{Leu(UUR)}$ (Wittenhagen and Kelley, 2002). The formation of this complex *in vitro* has been shown to be more efficient at physiological temperature, which implies a possibility of its presence *in vivo*, where it might interfere with multiple tRNA functions (Roy M et al, 2005).

Generally, the low steady-state levels of mutant tRNA could be considered as a result of its increased turnover or inefficient synthesis at various steps, and a defect in aminoacylation may further decrease the levels of functional tRNA.

Mutations affecting tRNA turnover

Structural defects caused by mutations may predispose tRNA to increased degradation. The A4269G mutation in tRNA^{Ile} leads to thermal instability and a dramatically decreased half-life of the tRNA as a result of susceptibility to nucleolytic attack (Yasukawa et al, 2000a). A reduced half-life of tRNA$^{Leu(UUR)}$ has been demonstrated in the case of the A3243G and T3271C mutations, both of which are associated with a decrease in aminoacylation and a deficiency of modification at the wobble position (Yasukawa et al, 2000b). Low steady-state levels of tRNA^{Asn} in the case of the G5703A mutation, which causes a conformational change, possibly accompanied by a defect in aminoacylation, were proposed to be a result of increased degradation (Hao and Moraes, 1997). This has not been confirmed, however, by studies on deacylated tRNA *in vitro* Nevertheless, aminoacylation could play a protective role against tRNA degradation *in vivo*. The affinity for the cognate aminoacyl-tRNA synthetase is likely to be a stabilizing factor that is involved in the control of tRNA levels (King and Attardi, 1993).

Mutations affecting tRNA synthesis

The processing and modification of tRNA involves its interaction with multiple enzymes that require correct tRNA structure. The mutations can affect any of these steps either directly at the site of interaction or indirectly by changing the overall structure of tRNA.

The mutations interfering with the processing of tRNA$^{Leu(UUR)}$ lead to a specific accumulation of the tRNA precursor, RNA19, containing the contiguous 16S rRNA, tRNA$^{Leu(UUR)}$ and ND1 mRNA genes (King et al, 1992). It was detected in the case of A3243G (King et al, 1992), A3302G (Bindoff et al, 1993), T3271C (Koga et al, 1995), and C3303T (Koga et al, 2003) mutations. In the case of other tRNAs, the precursors may be less abundant and/or less stable to allow them to be detected easily *in vivo*.

Although interference with RNA processing could be a common mechanism for many mutations, studies *in vitro* are so far restricted by the availability of the human processing enzymes. *In vitro* assays using partially purified mitochondrial tRNase Z demonstrated a complete blockage of 3´-processing by the A7445G mutation due to a change at the cleavage site resembling an anti-determinant for tRNase Z (Levinger et al, 2001). Later, the expressed long form of the enzyme (see section 2.3.5) was shown to process the substrate with the A7445G mutation with a reduced efficiency, producing aberrant cleavage products (Yan H et al, 2006). A decreased efficiency of tRNase Z cleavage was observed also for mutations in the acceptor stem of tRNA$^{Ser(UCN)}$: T7512C, T7511C and T7510C. Several mutations in tRNA^{Ile} (Levinger et al, 2003) and tRNA$^{Leu(UUR)}$ (Levinger et al, 2004) were found to impair 3´-processing efficiency as well.

The CCA addition step could be also sensitive to tRNA mutations, although the human tRNA nucleotidyltransferase has a rather loose substrate specificity with no conserved sequence requirement (Nagaike et al, 2001). Two mutations at similar positions in T-loops, A4317G in tRNA^{Ile} causing an aberrantly stable T-arm and A10044G in tRNA^{Gly} weakening tertiary interactions between T- and D-loops, significantly inhibited the catalysis of CCA addition, probably as a result of inappropriate positioning of the tRNA substrate on the enzyme (Tomari et al, 2003). From five studied mutations in tRNA$^{Leu(UUR)}$, only the C3303T mutation that destabilizes the first base pair in the acceptor stem had an effect on CCA addition, by decreasing the affinity of the enzyme for the mutant tRNA (Levinger et al, 2004).

Synthesis of post-transcriptional modifications could be potentially affected by point mutations directly, or as a consequence of tRNA structural alteration. In the first reported example, the C15990T mutation at position 36 in the anticodon of tRNA^{Pro} has been predicted to impair severely the neighbouring m^{1}G37 modification, which, on the other hand, might diminish a deleterious effect of the mutation at the strategic site (Brule et al, 1998). Either a direct or indirect effect of the A3243G mutation at position 14 in tRNA$^{Leu(UUR)}$ may account for a 50 % decrease in m^{2}G10 content (Helm et al, 1999). Furthermore, a global tRNA conformational change is likely to result in the loss of wobble base modifications found in tRNA$^{Leu(UUR)}$ with the A3243G and T3271C mutations, as well as in tRNA^{Lys} with the A8344G mutation (Yasukawa et al, 2000b; Yasukawa et al, 2000c). The taurine-containing modifications of uridine at the wobble position is essential for codon recognition and its lack has been demonstrated to result in severe loss of translation efficiency for AAA and AAG (lysine) and UUG (leucine) codons (Yasukawa et al, 2001; Kirino et al, 2004). A high density of

UUG codons in the ND6 mRNA has been suggested to explain complex I deficiency as the primary metabolic effect of the A3243G mutation (Jacobs 2003; Kirino et al, 2004). A unique suppression of the A3243G mutation by the G12300A mutation in the anticodon of tRNA[Leu(CUN)] (El Meziane et al, 1998b) could be explained by partial modification of the wobble base of the suppressor tRNA, enabling it to read the UUG codon in addition to the UUA codon (Kirino et al, 2006). The MELAS-associated mutations in tRNA[Leu(UUR)] have been shown to share a lack of the wobble base modification in tissues from patients (Kirino et al, 2005).

Mutations affecting tRNA aminoacylation

Aminoacylation could be potentially affected by mutations changing the identity elements or causing structural distortions of tRNA. So far, the results of studies on this as a possible pathological mechanism remain inconsistent. Decreased levels of the charged tRNA[Leu (UUR)] with the A3243G mutation were found in cybrid cell lines (El Meziane et al, 1998a; Chomyn et al, 2000), as well as in muscle biopsies from patients (Börner et al, 2000). However, these defects were variable, probably depending on the genetic background and epigenetic effects (Jacobs and Holt, 2000). The effect of the A8344G mutation could be also tissue-specific, as it was shown to cause an aminoacylation defect of tRNA[Lys] in osteosarcoma cybrids (Enriques et al, 1995) but not in the HeLa cellular background (Yasukawa et al, 2001) nor in tissues from patients (Börner at al, 2000). The mutation also did not change significantly the aminoacylation efficiency and extent (of the native tRNA) *in vitro* (Yasukawa et al, 2001; Sissler et al, 2004) and did not alter tRNA structure (Sissler et al, 2004).

Studies on tRNA transcript models suggest that a defect in aminoacylation may be not a primary molecular effect of the mutations in tRNA[Leu(UUR)] (Sohm et al, 2003), tRNA[Lys] (Sissler et al, 2004) and tRNA[Ile] (Kelley et al, 2000; 2001). Although residue A14 was identified as a strong leucine identity element of tRNA[Leu(UUR)], the A3243G mutation had a moderate effect on the efficiency of aminoacylation, whereas another mutation at the same position, A3243T, associated with a milder clinical disorder, produced a severe decrease (Sohm et al, 2003). No other mutations with locations either in the structured or floppy parts of tRNA[Leu(UUR)] lead to a dramatic defect in aminoacylation (Sohm et al, 2003), although for the T3271C mutation a defect in aminoacylation may be caused by the anticodon stem fragility (Wittenhagen et al, 2003).

In tRNA[Lys], two mutations linked to encephalopathies, G8313A and G8328A, dramatically impaired aminoacylation, whereas for nine other mutations the effects were either mild or absent (Sissler at al, 2004). Among them, no effect was found for the G8363A mutation, although it produced a drastic conformational change and a strong decrease of aminoacylation in cybrid cells (Bornstein et al, 2005). On the other hand, a significant decrease in

aminoacylation for the G8313A mutation was found also *in vivo* (Bacman et al, 2003).

In tRNA$^{\text{Ile}}$, ophthalmoplegia-associated mutations that cause structural fragility by creating A-C mispairs strongly reduced catalytic efficiencies of aminoacylation (Kelley et al, 2000). Moreover, the presence of mutant substrates inhibited charging of the wild-type tRNA$^{\text{Ile}}$. This could have deleterious effects in a heteroplasmic situation *in vivo* (Kelley et al, 2000). Two cardiomyopathy-related mutations in the T-arm of tRNA$^{\text{Ile}}$, C4320U and A4317G, had opposite effects on aminoacylation implying different connection to pathology (Kelley at al, 2001). An inherent fragility of the T-stem, however, may generally predispose tRNA$^{\text{Ile}}$ to the effects of pathological mutations (Kelley at al, 2001; Wittenhagen and Kelley, 2003).

Summary

The number of discovered pathogenic mtDNA mutations has exceeded 150 (www.mitomap.org) in less than 20 years. Almost 100 of them have been found in tRNA genes, consistent with the key role of tRNA in translation. Despite significant progress in recognition of tRNA point mutations as a cause of a wide spectrum of mitochondrial disorders, the understanding of their pathological mechanisms remains far behind. Even for the best studied mutations, e.g. the A3243G mutation in tRNA$^{\text{Leu(UUR)}}$ and the A8344G mutation in tRNA$^{\text{Lys}}$, the exact correlation with the clinical phenotype is not yet explained. A detailed understanding of the molecular effects of tRNA mutations is crucial for establishing their associations with specific pathologies. In the future, this knowledge will hopefully help to develop effective strategies for treatment of mitochondrial disorders.

2.5 Polyadenylation of stable classes of RNA

2.5.1 Functions of polyadenylation

Polyadenylation is as an important mechanism of regulation of RNA stability and translation, first found in eukaryotic mRNAs and generally assumed to protect their 3′-ends from exonuclease degradation. With increasing evidence of polyadenylation occurring also for stable RNA types (such as tRNA, rRNA, snRNA and snoRNAs) and in a variety of organisms, this process is emerging to be universal in the control of RNA metabolism.

Although the evidence for mRNA polyadenylation was discovered first in bacteria, its significance was ignored for many years, since poly(A) tails were short and only a small fraction of bacterial mRNAs carried them. However,

poly(A) tails have been demonstrated to play a role in degradation of bacterial mRNAs, probably serving as the targets for binding of RNA degradation complexes (O'Hara et al, 1995). A similar role of polyadenylation in mRNA degradation was observed in organelles, such as chloroplasts (Schuster et al, 1999) and mitochondria of *Trypanosoma brucei* (Ryan et al, 2003) and plants (Gagliardi et al, 2001). Yeast mitochondrial mRNAs, however, are not polyadenylated (Gagliardi et al, 2004). Thus, this mechanism is not evolutionarily conserved in mitochondria.

The role of polyadenylation of human mitochondrial mRNAs is not completely understood. Although polyadenylation may stabilize at least some of these mRNAs (Nagaike et al, 2005), shorthening of poly(A) tails after inhibiting of human mitochondrial poly(A) polymerase (hmtPAP), the enzyme responsible for their synthesis, did not result in an enhanced mRNA degradation (Tomecki et al, 2004). Polyadenylated aberrant mRNAs lacking stop codons were proposed to be rapidly degraded in a translation-dependent manner, possibly as a result of displacement of poly(A)-binding proteins by the translational machinery (Temperley et al, 2003). Recent studies, however, do not confirm this model. Although inhibition of mitochondrial translation stabilized some of the transcripts, in which complete stop codons are created by polyadenylation, no correlation of inaccurate processing of mRNA with translation-dependent deadenylation was observed (Piechota et al, 2006). A striking feature of RNA polyadenylation in human mitochondria is coexistence of both stably and internally polyadenylated transcripts, which implies opposite effects of poly(A) tails on RNA stability in the same organelle (Slomovic et al, 2005). Similar examples are known for polyadenylation of human ribosomal RNAs (Slomovic et al, 2006), as well as *T. brucei* mitochondrial RNAs (Kao and Read, 2005).

Stable polyadenylated RNAs, such as rRNAs, have also been found in several organisms. For example, in yeast, a small fraction of all rRNAs was found to be polyadenylated, which was increased in strains deficient for a ribonuclease of the nuclear exosome (Kuai et al, 2004). Similarly, in an *E. coli* strain lacking processing exonucleases, essentially all stable RNA species became polyadenylated (Li et al, 1998). These examples suggest that polyadenylation is involved in quality control of stable RNAs, which is discussed further in section 2.5.3. In bacteria, degradation of stable RNA usually occurs during starvation (Deutscher, 2003). It would be interesting to establish whether polyadenylation is involved in this mechanism, which is currently unknown.

Several human snRNAs, as well as 5S rRNA and cytoplasmic 7SL RNA, are also demonstrated to contain additional, usually single, adenine residues at their 3′-ends (Sinha et al, 1998). Such adenylation might play a role either in degradation or stabilization of these RNAs.

Functions of tRNA polyadenylation are described below.

2.5.2 Polyadenylation and degradation enzymes

Poly(A) polymerase I (PAP I) is the major polyadenylation enzyme in *E. coli*, accounting for synthesis of over 90% of the poly(A) tails, which are mainly homopolymers (O'Hara et al, 1995; Mohanty and Kushner, 1999). In the absence of PAP I, polynucleotide phosphorylase (PNPase) takes over this activity, generating highly heteropolymeric tails (Mohanty and Kushner, 2000). The degradation of bacterial mRNA is mediated by a large multiprotein complex, the RNA degradosome, which contains two 3´-5´ exonucleases, PNPase and RNase II (Carpousis et al, 1999). PNPase, as well as RNase R, are involved in degradation of stable RNAs (Li Z et al, 2002; Cheng and Deutscher, 2003).

In spinach chloroplasts, PNPase is suggested to function as both a poly(A) polymerase and an exonuclease, depending on the concentrations of inorganic phosphate and ADP (Yehudai-Resheff et al, 2001). Since no PAP I-like activity exists in cyanobacteria, which are believed to be closely related to the ancestor of chloroplasts, PNPase-mediated RNA polyadenylation and degradation could be considered as an evolutionarily more ancient mechanism (Rott et al, 2003). As mentioned in section 2.2.2, bacterial poly(A) polymerase and nucleotidyltransferase activities could have been interconverted later in evolution (Yue et al, 1996; Betat et al, 2004). In *Arabidopsis* chloroplasts, however, PNPase is unlikely to perform polyadenylation, which could be mediated by a prokaryotic-type PAP (Walter et al, 2002). Instead, a complex role for PNPase is suggested in the metabolism of all classes of chloroplast RNAs, including 3´-end processing of mRNA and 23S rRNA, as well as tRNA turnover.

Similarly, the PNPase identified in *Arabidopsis* mitochondria, AtmtPNPase (Perrin et al, 2004a), is also essential for several aspects of RNA metabolism, such as 3´-end maturation of *atp9* mRNA (Perrin et al, 2004a) and 18S rRNA, as well as degradation of 18S rRNA and its polyadenylated precursors (Perrin et al, 2004b). Moreover, it is proposed to play a special role in polyadenylation-dependent degradation of non-coding transcripts, such as tRNA and rRNA maturation by-products and antisense RNAs, which may compensate for a relaxed transcriptional control (Holec et al, 2006). No PNPase activity, however, has thus far been found in *T. brucei* mitochondria. In this organelle, degradation of polyadenylated RNAs could be performed by a hydrolytic exonuclease, such as RNase II (Ryan et al, 2003). In *Arabidopsis* mitochondria, AtmtRNase II is suggested to complete the 3´-processing by degradation of short remaining extensions (Perrin et al, 2004a).

Both PNPase (Piwowarski et al, 2003) and PAP (Tomecki et al, 2004) are present in human mitochondria. Significant shortening of the poly(A) tails of several mitochondrial mRNAs in the absence of PAP (Tomecki et al, 2004; Nagaike et al, 2005), plus their sequence homogeneity (Tomecki et al, 2004; Slomovic et al, 2005) suggests that they are synthesized by PAP. Although PNPase could act as a poly(A) polymerase *in vitro*, it has been suggested to function primarily as an exonuclease *in vivo* (Nagaike et al, 2005). Moreover,

PNPase might have other, not yet understood roles, such as regulation of cellular senescence (Piwowarski et al, 2003).

2.5.3 tRNA polyadenylation

Polyadenylation is suggested to be a major component in editing of overlapping tRNAs in metazoan mitochondria, which allows restoration of the discriminator base by a subsequent cleavage of the poly(A) tail followed by CCA addition (Yokobori and Pääbo, 1997). In support of this mechanism, overlapping tRNAs tend to carry A as the discriminator base (Yokobori and Pääbo, 1997). In land snail mitochondria, not only the discriminator bases but also nucleotides in the acceptor stem are subjected to editing, with possible involvement of polyadenylation (Yokobori and Pääbo, 1995). As observed in human mitochondria, polyadenylation might be involved in the repair of tRNAs, protecting them from degradation (Reichert and Mörl, 2000). Based on a predominant incorporation of A and C residues in the truncated tRNA molecules, such repair is also likely to be performed by tRNA nucleotidyltransferase (Reichert and Mörl, 2000). A similar activity may restore the overlapping discriminator base of human mitochondrial tRNA^{Tyr} by adding a missing A to the CC sequence (Reichert et al, 1998).

On the other hand, poly(A) tails were found in non-functional tRNA molecules, such as the tRNA^{Lys} pseudogene transcript in marsupial mitochondria (Dörner et al, 2001) and unedited larch tRNA^{His} precursor expressed in potato mitochondria, which cannot be correctly processed (Placido et al, 2005). Such molecules are likely to be targeted for degradation.

Polyadenylation appears to play an important role in tRNA quality control, as observed in bacteria (Li Z et al, 2002) and yeast (Kadaba et al, 2004). In bacteria, the low levels of a ts-mutant tRNA^{Trp} were found to be due to increased degradation of its precursor through polyadenylation (Li Z et al, 2002). In this model, PAP I is proposed to act as a scavenger enzyme that adds poly(A) tails to any exposed RNA 3′-end, as happens in the case of poor processing of abnormal RNA. Polyadenylated RNA is then targeted for degradation by 3′-5′ exonucleases, of which PNPase could be the major one.

A similar mechanism of polyadenylation is proposed for degradation of yeast initiator tRNA^{Met} lacking the m^{1}A58 modification, which is critical for its structural stability (Kadaba et al, 2004). The misfolded pre-tRNA is polyadenylated by the nuclear TRAMP (Trf4p/Air2p/Mtr4p polyadenylation) complex, which contains a novel poly(A) polymerase Trf4p (LaCava et al, 2005; Vanacova et al, 2005). This complex stimulates RNA degradation by the exosome, facilitated by the RNA helicase Mtr4p (LaCava et al, 2005). The aberrant RNA structure may be specifically recognized by contribution of the RNA-binding proteins Air1p or Air2p, either of which is required for the activity of the complex (Vanacova et al, 2005). However, substrate specificity for this pathway includes also rRNA and snoRNA precursors, as well as cryptic unstable

transcripts (CUTs) created by RNA polymerase II (LaCava et al, 2005; Wyers et al, 2005). Therefore, this could be a general mechanism of quality control of noncoding RNAs, possibly existing also in other eukaryotes (Wyers et al, 2005).

3. AIMS OF THE STUDY

Mitochondrial tRNA$^{Ser(UCN)}$ is a hot spot for deafness-associated mutations. The aim of my work was a detailed characterization of one of them, the 7472insC mutation, in a cultured human cell model, in order to establish its precise molecular mechanism and to compare its phenotypic effects with another deafness-associated mutation in the same tRNA. The specific aims of my study were:

1) to characterize the molecular and biochemical phenotype of the 7472insC mutation in 143B osteosarcoma cybrid cell lines

2) to determine the effect of the mutation on the structure and function of tRNA$^{Ser(UCN)}$

3) to test the effect of EF-Tu overexpression as a complementation strategy

4) to identify the exact steps of tRNA biosynthesis affected by the mutation

An additional series of experiments on human mitochondrial tRNA polyadenylation has been conducted during my studies. The aim of this on-going work has been to characterize the mechanism of polyadenylation-dependent degradation of aberrant tRNAs, which I provide in summary form, as unpublished results.

4. MATERIALS AND METHODS

This chapter summarizes general techniques and also supplements some details of specific methods used in the work. Full details are provided for methods not described fully in the published papers of this thesis.

4.1 Mammalian cells and cell culture

4.1.1 Cell lines and culture media

Cybrid cell lines containing mtDNA from a 7472insC patient were generated by Tiranti et al (1995) in the 143B osteosarcoma nuclear background by fusion of 143B rho^0 cells with patient myoblasts. The donor had 97% of 7472insC mutant mtDNA in muscle, which was associated with severe clinical symptoms. Resulting cell clones were homoplasmic for either mutant (clones 14, 30, 31, 32, 33, 38, 47) or wild-type (clones 34, 43) mtDNA. In studies on tRNA, line 47 (mutant) and line 43 (wild-type) were mostly used.

Cells were routinely cultured in DMEM medium containing 4.5 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml uridine, 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passaged weekly by trypsinization.

4.1.2 Growth assays

Confluent cells were washed in glucose-free medium after trypsinization and seeded in 96-well plates at 1/50 dilution in 200 μl medium containing either 4.5 g/l glucose or 0.9 g/l galactose. Estimation of viable cell number using MTT was performed as described by Wilson (1992). Cells were incubated in 200 μl medium containing 10 mM Hepes pH 7.4 with 2 mg/ml MTT (Sigma) for 3 h at 37 °C in the dark. Cells were then lysed in 200 μl DMSO followed by addition of 25 μl of Sorensen's buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). OD_{540} was measured using an ELISA plate reader (Labsystems). The initial cell density was estimated by the same method after the cells were attached to the plate (about 4 h) and cell growth was then observed over the following 4 days. Cell doubling times were calculated from the linearized growth curves and the relative ratio of

growth rates on galactose and glucose medium was estimated for each cell-line in each experiment.

4.1.3 Transfection and cloning

Cells were grown on 9 cm plates to 50-60% confluence. Transfection was carried out by vector pcDNA3.1(-) Myc/His A (Invitrogen) with or without EF-Tu insert created by J.N. Spelbrink (IMT, University of Tampere), using LipofectAMINE (Invitrogen) as described in (II). Cells were placed under Geneticin (Invitrogen) selection, and individual clones were picked and grown up. The presence of the construct was tested by PCR and RT-PCR, and the expression level was detected by Western blotting (see section 4.6.2).

4.2 Bacterial cell culture

Competent cells of *E. coli* strain XL1 Blue were cultured in LB medium (Sambrook et al, 1989) or on LB agar plates with selective antibiotics. For screening by dot hybridization, bacterial colonies were cultured on 96-well plates. Samples of bacterial culture were lysed on nylon membrane as described by Sambrook et al (1989) and hybridized with a ^{32}P-labelled oligonucleotide (see section 4.3.2).

4.3 Miscellaneous RNA and DNA manipulations

4.3.1 Oligonucleotides

Table 4.1 (a) *PCR and sequencing*

Name	Sequence 5´-3´	MITOMAP position, np[1]	Specificity
FR31	CTT CCC ACA ACA CTT TCT CGG	7178-7198	COXI
FR32	GTA AAG GAT GCG TAG GGA TG	7840-7821	COXII
MT11	GAA CCC TCC ATA AAC CTG GAG	7362-7382	COXI
MT12	TGC GCT GCA TGT GCC ATT AAG	7602-7582	COXII
FR6	GGT GCA GCC GCT ATT AAA GGT TCG T	3013-3037	16S
FR7	CCG ATC AGG GCG TAG TTT GAG TTT G	3698-3674	ND1
18S-F	TAC CTG GTT GAT CCT GCC AG		18S rDNA[2]
18S-R	TCG GGA GTG GGT AAT TTG C		18S rDNA[2]
M13-F	GTT TTC CCA GTC ACG AC		M13
M13-R	CAG GAA ACA GCT ATG AC		M13

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Table 4.1 (b) *Northern, dot and solid phase hybridization*

Name	Sequence 5´-3´	MITOMAP position, np[1]	Specificity
Ser11	AAG GAA GGA ATC GAA CCC CCC AAA GCT G	7451-7458	tRNA[Ser(UCN)]
Ser12	CCA ACC CCA TGG CCT CCA TGA CTT TTT C	7487-7514	tRNA[Ser(UCN)]
Ser-Bio	AGC CAA CCC CAT GGC CTC CAT GAC TTT TTC-Biotin	7485-7514	tRNA[Ser(UCN)]
Leu21	GTT TTA TGC GAT TAC CGG GC	3263-3244	tRNA[Leu(UUR)]
Lys	AAA GGT TAA TGC TAA GTT AGC	8304-8324	tRNA[Lys]
Gln	GAA TCG AAC CCA TCC CTG AG	4341-4360	tRNA[Gln]
Tyr	ATT TAC AGT CCA ATG CTT CAC TC	5857-5879	tRNA[Tyr]
5S1	GGG TGG TAT GGC CGT AGA C		5S rRNA[3]
Ser31	TAG TCG GCA GGA TTC GAA CCT GCG TGG		cytosolic tRNA[Ser(UCH)][4]

Table 4.1 (c) *RT-PCR and minisequencing on tRNA[Ser(UCN)]*

Name	Sequence 5´-3´	MITOMAP position, np[1]
Cser1	TGG CCT CCA TGA CTT TTT C	7496-7514
Cser2	ATG GGG TTG GCT TGA AAC	7496-7479
Cser3	CTT GAA ACC AGC TTT GGG GGG	7486-7467
Cser4	AAG GAA GGA ATC GAA CCC CCC	7451-7471
Cser5	CAA AAA AGG AAG GAA TCG AAC C	7446-7467
Cser6	GAA AAA GTC ATG GAG GCC ATG	7514-7494

Table 4.1 (d) *Pre-tRNA[Ser(UCN)] constructs*

Name	Sequence 5´-3´	MITOMAP position, np[1]
Sp6L1	**ATTTAGGTGACACTATAGAATAC** *GAATTC*-TAAGATATATAGGATTTAG	**Sp6**+*EcoRI*+ 5´-leader (7585-7559)
Sp6S1	**ATTTAGGTGACACTATAGAATAC** *GAA*	
SerCCA1	tgg CAAAAAAGGAAGGAATCGAAC	tgg+(7446-7467)
SerTCCA	tgg**a** CAAAAAAGGAAGGAATCGAAC	tgg**a**+(7446-7467)
Trail31	**AGAACCCGTATACATAAAATCTAGA**-CAAAAAAGGAAGGAATCGAACCC	**3´-trailer (7421-7445)**+ (7446-7468)

[1]Human mitochondrial DNA revised Cambridge reference sequence (www.mitomap.org)

[2] Gonzalez and Schmickel (1986)

[3] Nielsen et al (1993)

[4] Krupp et al (1988)

4.3.2 RNA extraction and analysis by Northern hybridization

Total RNA was extracted from semiconfluent cells using the Trizol reagent (Life Technologies), as recommended by the manufacturer. RNA isolated for analysis of aminoacylation was dissolved in 0.1 M NaAc pH 5.2. To deacylate tRNAs, 1.5 volumes of 0.5 M Tris-HCl pH 9.0 were added and samples were heated for 10 min at 75 °C, followed, in some experiments, by another incubation for 30 min at 37 °C. RNA was separated either on 1.2% agarose-formaldehyde (I) or 12% polyacrylamide-7M urea/TBE (II) gels. Aminoacylation was analysed on 6.5% polyacrylamide-7M urea/ 0.1M NaAc pH 5.2 gels (Enriquez and Attardi, 1996). Agarose gels were capillary blotted to Hybond-Nylon membranes (Amersham Biosciences). Polyacrylamide gels were electroblotted in TBE or TAE to Zeta-Probe GT membranes (Bio-Rad).

Oligoprobes were 5′-labelled with $[\gamma\text{-}^{32}P]ATP$ (3000 Ci/mmol, Amersham Biosciences) using T4 polynucleotide kinase, and purified on mini Quick Spin Oligo Columns (Roche). PCR probes were randomly labelled in the presence of $[\alpha\text{-}^{32}P]dCTP$ (3000 Ci/mmol, Amersham Biosciences) using the Oligolabelling kit (Amersham Biosciences). Riboprobes were labelled and hybridized as described in (I).

Northern hybridization was performed overnight at 42 °C in 50 mM PIPES, 100 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, 5% SDS, 100 μg/ml salmon sperm DNA, pH 6.8. Washes were performed at 42 °C twice for 6 min in 2xSSC, 0.1% SDS and for 12 min in 1xSSC, 0.1% SDS. Membranes were stripped by boiling in 0.5% SDS and reprobed. Signals were analysed by autoradiography and quantified by phosphorimaging and densitometry (Molecular Dynamics).

4.3.3 DNA extraction and analysis by Southern hybridization

DNA was extracted for Southern analysis as described in (I). Briefly, cells from one confluent 9 cm plate were collected, resuspended in water and treated with boiled RNase A followed by incubation in guanidine hydrochloride buffer containing proteinase K. DNA was precipitated using isopropanol and digested three times with *Hin*dIII, followed by phenol/chloroform extraction and ethanol precipitation after each digestion.

DNA was separated on 0.7% agarose gel and capillary blotted to MagnaCharge nylon membrane (Micron Separation, Westborough, USA). Hybridization was performed overnight at 65 °C in 1 mM EDTA, 7% SDS, 0.5 M sodium phosphate buffer pH 7.2 (Church and Gilbert, 1984). PCR probes for ND1 and 18S rDNA were prepared as described in (I) and randomly labelled as described above. Washes of 20 min each were performed at 65 °C: twice in 3xSSC, 0.1% SDS and once in 0.3xSSC, 0.1% SDS. Signals were analysed as described above.

4.3.4 PCR and RT-PCR

PCR reactions were performed on crude DNA extract prepared by incubation of cells in K-buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0, 2.5 mM MgCl$_2$, 0.5% Tween 20, 100 µg/ml proteinase K) for 45 min at 56 °C, followed by inactivation of proteinase K for 10 min at 90 °C. PCR reactions were carried out under optimized conditions. Generally, mtDNA fragments were amplified in 25 µl of buffer with 1.5 mM MgCl$_2$ containing 0.2 mM dNTPs, 0.2 µM of each primer and 0.6 u of Dynazyme DNA polymerase (Finnzymes) at 30 cycles of annealing at 55 °C for 1 min and extension at 72 °C for 3 min with final extension at 72 °C for 15 min. Circularized tRNAs were amplified in 25 µl reactions with 0.25 mM dNTPs and 0.4 µM of each primer using short cycles (92 °C for 2 min; 30 cycles of 94 °C for 30 sec; 55 °C for 30 sec; 72 °C for 30 sec; final extension at 72 °C for 5 min).

To analyse transgene expression, RT-PCR was performed on total RNA from which any traces of DNA were removed, as follows: RNA extracted from a 9 cm plate was incubated in 100 µl of buffer (40 mM Tris-HCl pH 7.5, 6 mM MgCl$_2$, 1 mM DTT) containing 1 u of RNase free DNase I (Roche) for 1 h at 37 °C. RNA was extracted with equal volumes of acid phenol pH 4.0 and chlorophorm/isoamyl alcohol (25:1) saturated with 0.1 M Tris-HCl pH 8.0 and isopropanol precipitated. For the RT step, 10 µg of RNA was denatured by heating and chilling on ice and incubated for 1 h at 37 °C in 20 µl of the reaction mixture containing 0.00125 u/µl of random hexamers (Pharmacia), 10 mM DTT, 0.5 mM dNTPs, 10 u of RNAguard (Pharmacia) and 200 u of M-MLV RTase (Gibco BRL) in 1x first strand buffer (Gibco BRL). After denaturation by heating and chilling on ice, 2 µl of the cDNA product was used for PCR amplification.

For RT on circularized tRNA, 1 µl of template was used in a 20 µl reaction containing 0.5 µM primer and 0.25 mM dNTPs in 1x first strand buffer (MBI Fermentas). After annealing of the primer by heating the reaction mixture for 2 min at 92 °C and cooling for 10 min at room temperature and for next 10 min on ice, 20 u of M-MLV RTase (MBI Fermentas) was added and the reaction was incubated at 37 °C for 1h. 2 µl of the cDNA product was used for PCR amplification.

4.3.5 Molecular cloning

PCR products were cloned using the TOPO TA cloning kit (Invitrogen) according to manufacturer's recommendations. X-gal (MBI Fermentas) was used for blue/white screening. Since inserts of tRNA$^{\text{Ser(UCN)}}$ sequence produced clones of both colours, they were screened by dot hybridization with oligonucleotide Ser11. Plasmid DNA was isolated using the NucleoSpin Robot-96 plasmid kit (Macherey-Nagel).

4.3.6 DNA sequencing

DNA sequencing was performed using the BigDye terminator kit (Applied Biosystems) with primers designed for mtDNA or M13 universal primer. The products were analysed by capillary electrophoresis on an ABI 310 Genetic Analyzer using manufacturer's software. SNP minisequencing was performed using ABI Prism SNaPshot ddNTP primer extension kit (Applied Biosystems).

4.4 tRNA structural and functional analysis

4.4.1 tRNA sequencing and structural probing

Total RNA was extracted from semiconfluent cells in amounts of approximately 1000 A_{260} units (i.e. from 200 9 cm plates) for primary sequence determination or 500 A_{260} units (i.e. from 100 9 cm plates) for analysis by partial RNase digestion. tRNAs were deacylated by incubation of total RNA in 20 mM Tris-HCl pH 9.0 at 37 °C for 2 h, after which pH was adjusted to 7.5 by the addition of 3M NaAc pH 5.2. Small RNAs were isolated by fractionation of total RNA on a 1x45 cm DEAE-Sepharose fast flow column (Amersham Biosciences) as described by Yasukawa et al (2000). RNA was bound to the column in 250 mM NaCl, 20 mM Tris-HCl pH 7.5, 8 mM MgCl$_2$ and eluted by a linear gradient to 500 mM NaCl, 20 mM Tris-HCl pH 7.5, 16 mM MgCl$_2$. Collected fractions were monitored for tRNA$^{Ser(UCN)}$ by dot hybridization with oligonucleotide Ser11 and concentrated on Centriprep 30 columns (Amicon) before precipitation. tRNA$^{Ser(UCN)}$ was finally purified by solid phase hybridization to 3′-biotinylated oligonucleotide Ser-Bio as described by Wakita et al (1994) under the following conditions. The biotinylated probe was bound to streptavidin agarose (Gibco BRL) in 100 mM Tris-HCl pH 7.5 for 1h at room temperature and extensively washed with 10 mM Tris-HCl pH 7.5. The probe was annealed to tRNA using a temperature gradient from 62 to 37 °C in a buffer (1.2 M NaCl, 30 mM Tris-HCl pH 7.5, 15 mM EDTA) and washed with a 0.5x dilution of this buffer. tRNA was eluted several times for 5 min at 65 °C each, with the buffer diluted 1:60, precipitated and purified by gel electrophoresis.

Purified tRNAs were sequenced by a combination of methods of partial RNase digestion (Donis-Keller, 1980) and two-dimensional TLC (Kuchino et al, 1987) with modifications as described by Yasukawa et al (2000) and in (II). For partial RNase digestion, the tRNA was labelled at the 5′-end with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Biosciences) using T4 polynucleotide kinase (MBI Fermentas) or at the 3′-end with [^{32}P]pCp (3000 Ci/mmol, Amersham Biosciences) using T4 RNA ligase (MBI Fermentas). Before 5′-end labelling, the tRNA was dephosphorylated by bacterial alkaline phosphatase (MBI Fermentas) followed by phenol extraction and ethanol precipitation. Labelled

tRNAs were purified on a 12% polyacrylamide-7M urea/ TBE gel, eluted by 0.5 M ammonium acetate pH 5.2, 0.1% SDS, 0.1 mM EDTA, and ethanol precipitated in the presence of 20 μg of yeast carrier tRNA (Sigma). Partial digestion was carried out under denaturing conditions in 30 mM sodium citrate pH 4.5, 1 mM EDTA, 8 M urea, 0.02% xylene cyanol and bromphenol blue at 55 °C. Digestion with RNase CL was performed in 20 mM Tris-HCl pH 7.5, 8 M urea, 0.02% xylene cyanol and bromphenol blue. Alkaline digestion was performed in 50 mM Na_2CO_3 pH 9.0 at 95 °C. The digested tRNA fragments were separated on 12% or 15% polyacrylamide-7M urea/ TBE gel. At least 5000 cpm of tRNA per reaction was assayed with the following RNases:

Table 4.2. *RNases used for partial digestion of tRNA*

RNase	Source	Nucleotide specificity	Comments
T1	USB	G	
U2	Amersham Biosciences	A > G	
Phy M	Amersham Biosciences	A; U	Enzyme no longer available
CL	Sigma	C	
A	USB	C; U	
ONE	Promega	None-specific	
T2	Sigma	None-specific	

For the chromatographic analysis of 5´-postlabelled nucleotides, the purified tRNA was incubated at 95 °C for 1.5 min in water to produce random limited hydrolysis. The 3´-half fragments were 5´-labelled and separated on a 15% polyacrylamide-7M urea/ TBE gel containing 10% glycerol. Each band was cut out, extracted from the gel and digested completely by 1 μg of RNase P1 in 20 mM ammonium acetate pH 5.2 at 37 °C overnight. An aliquot of each digestion was applied to a 10 x 10 cm TCL plate and analysed with two solvent systems as described by Yasukawa et al (2000) and in (II).

Structural probing was performed under non-denaturing conditions at 37 °C on tRNA refolded in renaturation buffer (150 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5) as described in (II).

4.4.2 Aminoacylation assay (oxidation-circularization)

The oxidation-circularization assay developed by Börner et al (2000) was used with modifications as described in (II) (Fig. 4.1), either on total RNA or on small RNAs isolated on DEAE-Sepharose fast flow column (Amersham Biosciences).
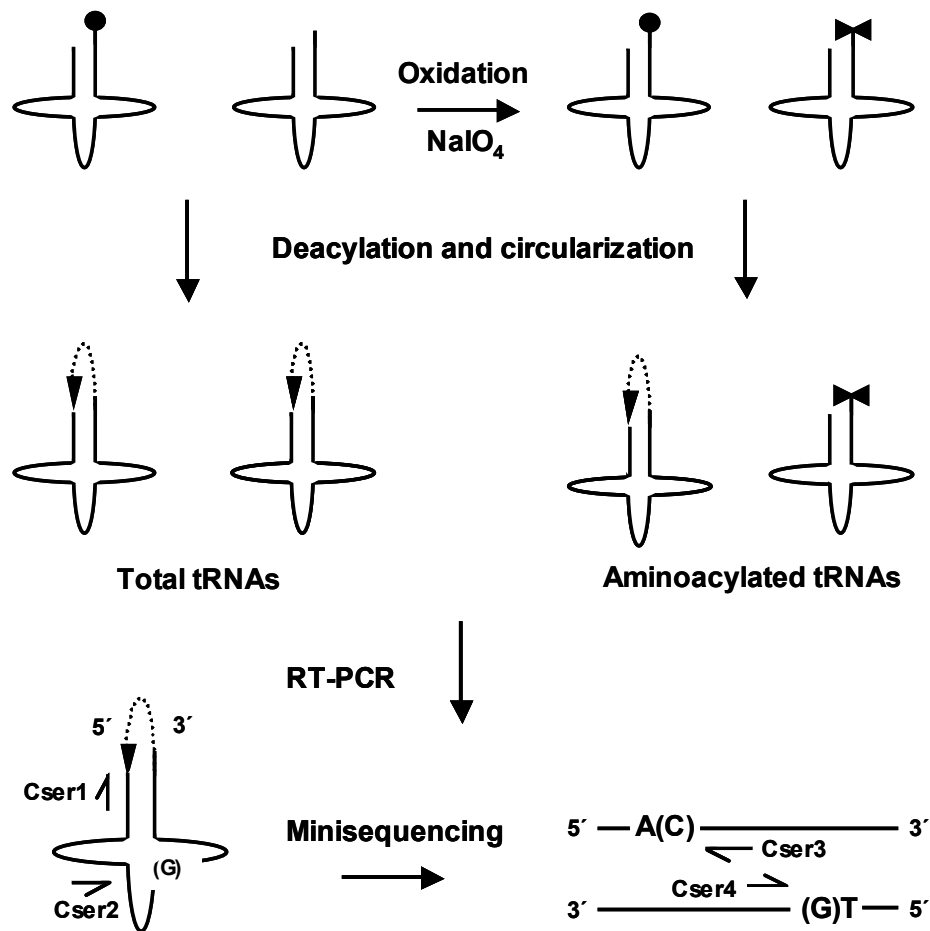
Figure 4.1. *Principle of the oxidation-cirularization assay. tRNA not protected by amino acid is oxidized by periodate (double triangles), whereas only aminoacylated tRNA (filled circles) can be circularized after subsequent deacylation (dashed arrows) and RT-PCR amplified. The relative extent of aminoacylation of mutant (letters in parentheses) and wild-type tRNA present in the same reaction mixture can be then estimated by fluorescent minisequencing. Two minisequencing primers designed for coding and non-coding strands were used.*

Before the assay, total RNA from wild-type and mutant cells was mixed in various proportions. About 25 μg of total mixed RNA used in two sets of two reactions was either ethanol precipitated or bound to DEAE-Sepharose column in a buffer (50 mM NaAc pH 5.2, 10 mM MgCl₂) with 100 mM NaCl, eluted by the same buffer containing 900 mM NaCl, and precipitated in aliquots in the presence of 20 μg/ml glycogen (Boehringer Mannheim). In the first reaction, RNA was resuspended in 32 μl of circularization buffer containing 10 μl of 4x buffer (200 mM Tris-HCl pH 7.7, 40 mM MgCl₂, 0.4 μg/μl BSA) and incubated for 10 min at 75 °C followed by 30 min at 37 °C to deacylate tRNAs. In the second reaction, RNA was resuspended in 20 μl of oxidation buffer (10 mM NaAc pH 5.2, 10 mM MgCl₂, 1 mM EDTA, 15 mM β-mercaptoethanol) and oxidized by adding 20 μl of 65 mM NaIO₄ and incubation for 25 min at room temperature in the dark. The reaction was stopped by adding an equimolar

amount of glucose. RNA was ethanol precipitated twice from a volume of 400 μl in the presence of 20 μg/ml glycogen and deacylated as described above. Deacylated tRNAs from both reactions were circularized by adding 0.6 μl of 10 mM ATP, 6 μl DMSO, 40 u T4 RNA ligase (MBI Fermentas) and incubation for 2 h at 37 °C. Circularized tRNAs were reverse-transcribed with primer Cser1 and PCR-amplified with primers Cser1 and Cser2. The products were purified by gel electrophoresis and analysed by minisequencing with primers Cser3 and Cser4.

4.4.3 tRNA half-life and synthesis measurements

The various experiments were performed as described in (II). For half-life measurements, cells seeded on 6 cm plates were treated with 250 ng/ml EtBr for times of between 4 and 24 h. For tRNA synthesis measurements, cells seeded on 9 cm plates were treated with 250 ng/ml EtBr for two days, then seeded on 6 cm plates and cultured in fresh medium changed daily up to 7 days. Isolated RNA was analysed by Northern hybridization and the signals normalized against 5S rRNA.

4.5 tRNA processing

4.5.1 In vivo processing analysis

6 μg of total RNA was deacylated, circularized and RT-PCR amplified as described in 4.4.2. Gel-purified products were cloned as described in 4.3.5 and sequenced with M13 forward primer.

4.5.2 Pre-tRNA constructs

The constructs with or without the 7472insC mutation were prepared as described in (III).

(a) The constructs with a 71 bp 5′-leader sequence preceded by an *Eco*RI site and with a mature 3′-end were created by PCR amplification of a mtDNA fragment containing tRNA$^{Ser(UCN)}$ with primers SP6L1 to introduce the SP6 promoter sequence and SerCCA1 to introduce the 3′-terminal CCA sequence. The constructs were reamplified with primers SP6S1 and SerCCA1 using Pfu DNA polymerase (Promega) to create blunt ends, cloned and verified by sequencing.

(b) The constructs with the 3′-trailer were prepared by amplification of constructs (a) with primers SP6S1 and Trail31 to introduce a 25 bp trailer sequence.

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(c) The constructs with a misprocessed 3′-end were created by amplification of constructs (a) with primers SP6S1and SerTCCA to introduce the 3′-terminal TCCA sequence.

The constructs (a-c) were transcribed *in vitro* from SP6 promoters, as follows: 0.5 pmol of DNA template was incubated for 1 h at 37 °C in 20 µl of 1x transcription buffer (MBI Fermentas) containing 20 u RNasin (MBI Fermentas), 0.5 mM ATP, GTP and UTP, 20 µM CTP, 50 µCi [α-^{32}P]CTP (3000 Ci/mmol, Amersham Biosciences) and 20 u SP6 polymerase (MBI Fermentas). DNA template was then removed by addition of 1 u RNase free DNase I (Roche) per µg of DNA template and incubation for 15 min at 37 °C. The reaction products were phenol extracted and purified by gel electrophoresis as described in (III).

(d) The constructs with mature 5′-ends were prepared by L.L. Levinger (York College/CUNY) by ligation of two ½-tRNA oligonucleotides followed by 5′-end labelling as described in Levinger et al (2001).

4.5.3 In vitro processing analysis

Processing assays were performed with partially purified HeLa cell RNase P (Nadal et al, 2002) and mitochondrial tRNase Z (Levinger et al, 2001) under the conditions described in (III). Enzyme preparations were kindly supplied by these authors. Briefly, the processing reactions were carried out on the refolded substrates with the enzymes at various dilutions. The reactions were stopped by incubation in an equal volume of pronase-containing buffer. The products were phenol extracted, ethanol precipitated and analysed on 5% polyacrylamide-7 M urea/ TBE gels. The termini of the products extracted from the gel were identified by circularization, RT-PCR, cloning and sequencing as in 4.5.1. For quantification of the relative efficiency of 5′-processing, the mutant and wild-type substrates mixed at equal proportion were processed in the same reactions with RNase P at various dilutions. The substrates and products were gel extracted, reverse-transcribed with primer Cser5, PCR-amplified with primers Cser5 and Cser6, and the ratio of mutant and wild-type molecules quantified by minisequencing with primers Cser3 and Cser4. The relative processing efficiency was determined from the ratio of mutant and wild-type molecules in products against this ratio in the remaining substrates for each reaction.

4.6 Analysis of mitochondrial proteins

4.6.1 SDS-PAGE

[^{35}S]-methionine labelling of mitochondrial proteins was performed on cells grown on 6 cm plates to 60-70% confluence. Cells were washed and then

incubated in DMEM lacking methionine for 10 min at 37 °C. Cells were washed again and incubated in the presence of 10 µg/ml emetine for 10 min at 37 °C. 20 µCi/ml of L-[^{35}S]-methionine (1000 Ci/mmol, Amersham Biosciences) were added and cells were incubated for 30 min at 37 °C. Cells were washed twice with DMEM, collected by trypsinization, washed with PBS by centrifugation and suspended in 75 µl of 1.5% lauryl maltoside (Fluka) in PBS containing 2.5 mM PMSF. After incubation for 30 min on ice, the lysates were centrifuged for 2 min at 14 000 g$_{max}$ and supernatants collected. The translation products were analysed by SDS-PAGE on 16% polyacrylamide gels (Schägger and von Jagow, 1987).

4.6.2 Western blotting

The procedures were carried out as described by Spelbrink et al (2000) and in (II). Briefly, cells were lysed in Triton-X100-containing buffer, proteins separated by SDS-PAGE on a 10% polyacrylamide gel and wetblotted to Hybond-C extra nitrocellulose membrane (Amersham Pharmacia). Blots were blocked in TBS-T buffer containing 0.1% Tween and 5% fat-free milk powder, washed and incubated with primary antibody detecting EF-Tu or PAK1 (p21-associated kinase) (see (II) for references) used as a loading control. Blots were then washed and incubated with horseradish peroxidase-conjugated secondary antibody. Blots were washed again, treated with luminol solution and exposed to X-ray film.

5. RESULTS

5.1 Molecular and biochemical phenotype of the 7472insC mutation in cybrid cells (I)

The 7472insC mutation was previously found to cause a moderate decrease in complex I activity and oxygen consumption (Tiranti et al, 1995). My task was to characterize the phenotype of the mutation in detail and to compare it to the effects of the A7445G mutation previously studied in lymphoblastoid cells (Reid et al, 1997; Guan et al, 1998). In my studies, seven osteosarcoma cybrid cell lines homoplasmic for the 7472insC mutation were compared with two cell lines homoplasmic for the wild-type mtDNA from the same patient (i.e. identical to the mutant mtDNA at every nucleotide apart from the C insertion at np 7472).

5.1.1 Steady-state levels of tRNA$^{Ser(UCN)}$

The levels of mutant tRNA$^{Ser(UCN)}$ were estimated by Northern hybridization with two oligonucleotides, Ser11 and Ser12, which gave identical results when normalized against mitochondrial ND1 mRNA as well as cytosolic G3PDH mRNA. Whereas the levels of tRNA$^{Leu(UUR)}$ were not changed, the levels of tRNA$^{Ser(UCN)}$ were significantly reduced in all mutant clones (Fig. 5.1a, b). With small variations, the average decrease of the mutant tRNA was ~35% of the control level, i.e. very similar to the values found in case of the A7445G mutation (Reid et al, 1997; Guan et al, 1998). Since the A7445G mutation was associated also with a significant decrease in the level of ND6 mRNA cotranscribed from the L-strand (Guan et al, 1998), I next checked the pattern of the L-strand transcripts in the 7472insC cybrids using an ND6-specific riboprobe. The abundance of the L-strand RNA precursors and ND6 mRNA in the mutant cells was not consistently altered, with the exception of one clone (line 14) were the ND6 mRNA was strongly induced (Fig. 5.2). This cell line had also a higher level of tRNA$^{Ser(UCN)}$, namely ~55% of the control value. In order to investigate whether this might reflect a second site mutation that could influence overall RNA processing efficiency, line 14 was sub-cloned and the level of tRNA$^{Ser(UCN)}$ was estimated in individual subclones (unpublished results, Fig. 5.1c). In 11 subclones, these values varied from ~25% to ~65%, however, most of them were not significantly different from the average levels observed in other cell lines. The mtDNA from two subclones with the lowest and the highest

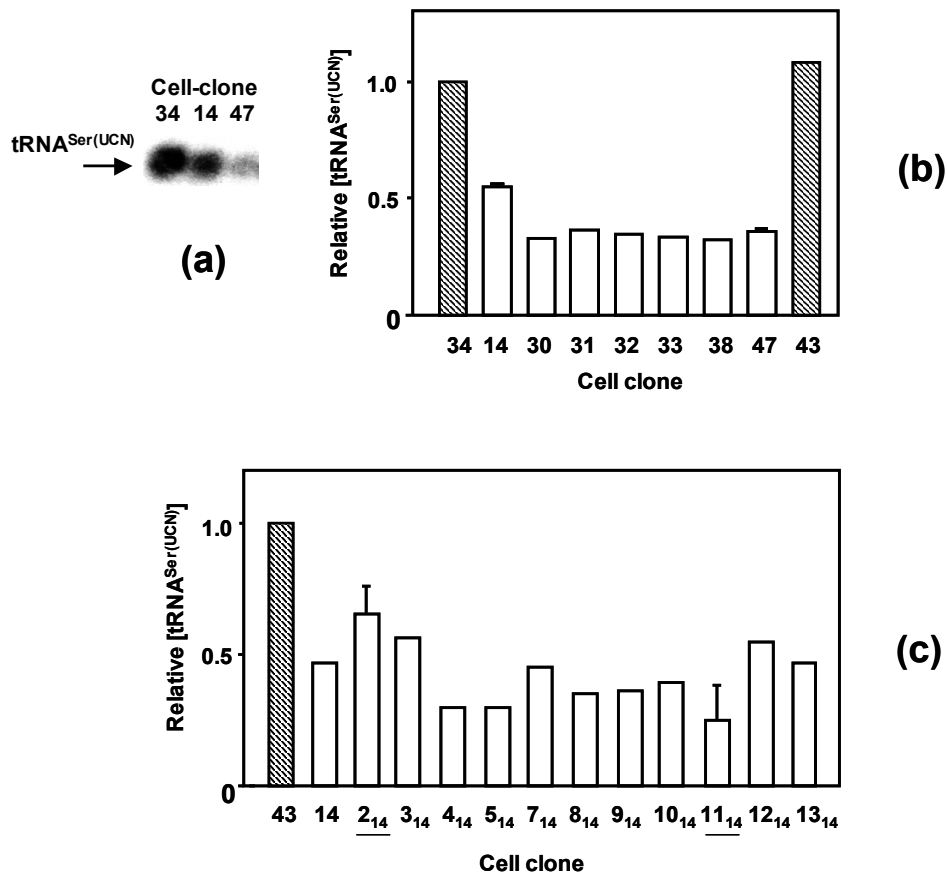levels of tRNA$^{Ser(UCN)}$ was compared by complete mtDNA sequencing, but no obvious changes were found.

Figure 5.1. *Steady-state levels of tRNA$^{Ser(UCN)}$ in the 7472insC cybrid cell lines. (a) An example of Northern blot for a control (34) and two mutant (14, 47) cell clones probed with an oligonucleotide specific for tRNA$^{Ser(UCN)}$. The signals were normalized against ND1 mRNA and G3PDH mRNA (not shown). (b) Relative steady-state levels of tRNA$^{Ser(UCN)}$, normalized to that of control clone 34. Control clones 34 and 43 are shown as shaded bars. For clones 14 and 47, standard errors were estimated based on three experiments. Reprinted from original article (1), copyright (1999), by permission of Oxford University Press. (c) Relative steady-state levels of tRNA$^{Ser(UCN)}$ in subclones of clone 14, normalized to that of control clone 43 (shaded bar). Note that a different control clone than in (b) was used as a standard. Subclones with the highest (line 2$_{14}$) and the lowest (line 11$_{14}$) levels of tRNA$^{Ser(UCN)}$ are underlined. Standard errors for these clones were estimated based on three experiments.*

5.1.2 Growth on galactose medium

Galactose medium was used to check the ability of cells to grow under conditions of respiratory stress. The mutant clones had only a mild growth defect on this medium, observed previously also in cells with the A7445G mutation (Reid et al, 1997). The ratios of the relative growth rates on galactose medium

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versus normal medium varied slightly between individual clones including wild-type cell lines (Fig. 5.3a). For one of the clones (line 47), however, this ratio was significantly decreased.
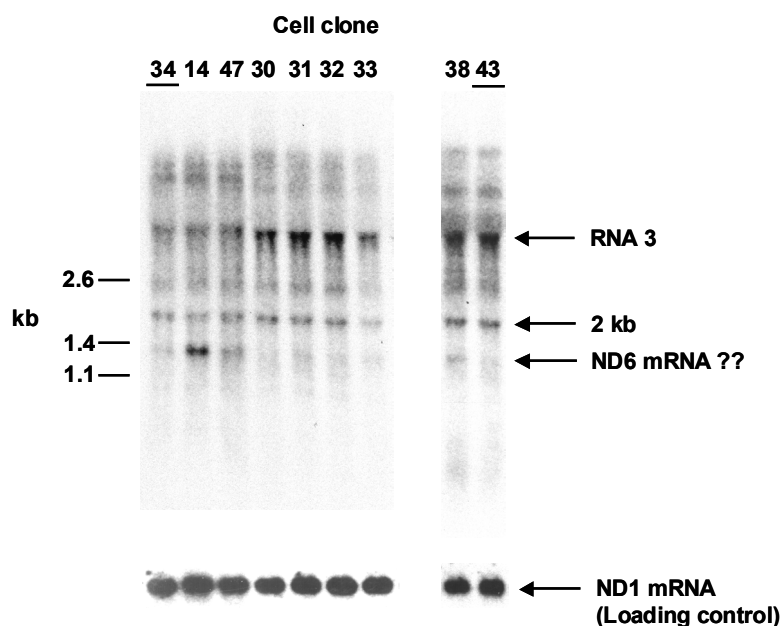

Figure 5.2. *Transcripts of the ND6 region in the 7472insC cybrid clones. The Northern blot was hybridized with a riboprobe specific for ND6 mRNA. The putative positions of the L-strand transcripts are indicated by arrows. ND1 probe was used as a loading control. The control clones 34 and 43 are underlined. Reprinted from original article (I), copyright (1999), by permission of Oxford University Press.*

5.1.3 mtDNA copy number

To explain the observed variations in the ability of cells to grow on galactose medium, I estimated the relative mtDNA copy numbers by Southern hybridization with probes for mitochondrial (ND1) and nuclear (18S) DNA (Fig. 5.3b, c). For clone 47, the growth defect under respiratory stress correlated with the lowest mtDNA copy number. On the other hand, clone 14 with the highest copy number and less decrease in tRNA$^{Ser(UCN)}$ level did not show any growth advantage. For wild-type clone 43, which did exhibit a slight growth advantage, the mtDNA copy number was low. For other clones, a weak positive correlation between mtDNA copy number and growth rates was observed.
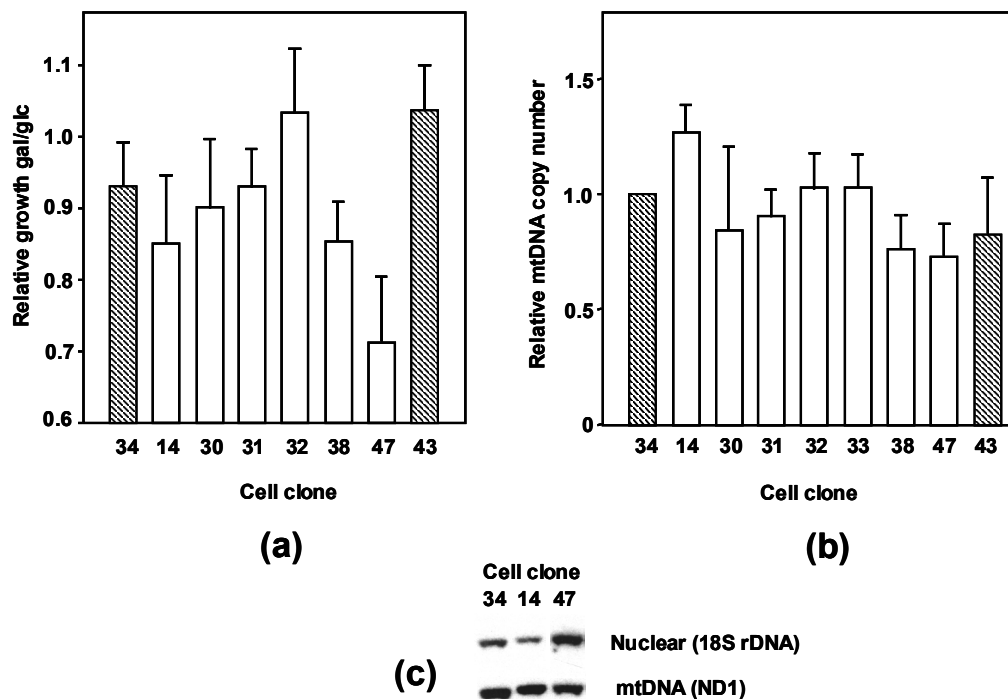
Figure 5.3. *Growth of the 7472insC cybrid clones on galactose medium and mtDNA copy number. (a) Relative growth rates calculated as a ratio of doubling times on glucose and galactose medium for each cell clone in each experiment. Control clones 34 and 43 are shown as shaded bars. (b) Relative copy numbers of mitochondrial and nuclear DNA estimated as a ratio of signals for ND1 and 18S rDNA probes, normalized to that of control clone 34. (c) An example of Southern blot for clones 34 (control), 14 (highest copy number) and 47 (lowest copy number). Reprinted from original article (I), copyright (1999), by permission of Oxford University Press.*

5.1.4 Mitochondrial protein synthesis

The effect of the mutation on mitochondrial protein synthesis was studied by pulse labelling of cells with [^{35}S]-methionine in the presence of emetine to inhibit cytosolic translation. Although there was no difference in the pattern of mitochondrial translation products, a small quantitative decrease was observed for most of the mutant clones (Fig. 5.4). For clone 47, this decrease was pronounced in the presence of doxycycline. Two previous studies on the A7445G mutation reported different effects on mitochondrial translation, which was either not detectably affected (Reid et al, 1997) or else decreased by ~55% (Guan et al, 1998).

5.1.5 The 7472insCC mutation

While the presence of homoplasmic 7472insC mutation in the cybrid cells was confirmed by direct sequencing across the tRNA$^{Ser(UCN)}$ region, a second C insertion at np 7472 was found in one of the clones (line 38). The level of this

additional mutation was estimated to be ~15% in clone 38 and ~10-20% in 18 subclones derived from this line. The presence of the mutation was not observed in the donor patient, although an additional A to C transition at np 7472 resulting in the same length change of homopolymeric C run was recently reported for patients with the 7472insC mutation (Pulkes et al, 2005; Cardaioli et al, 2006). In my study, the phenotype of clone 38 was not noticeably different from other clones.
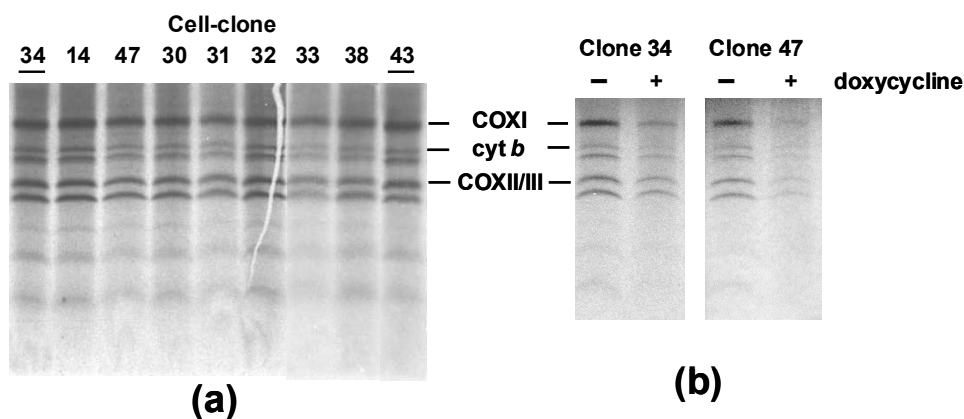

Figure 5.4. *Mitochondrial translation products in the 7472insC cybrid clones. (a) [^{35}S]-methionine-labelled mitochondrial proteins synthesized in the presence of emetine. The control cybrids 34 and 43 are underlined. (b) The effect of doxycycline (200 µg/ml) on mitochondrial translation in clones 34 (control) and 47 (mutant). Reprinted from original article (I), copyright (1999), by permission of Oxford University Press.*

5.2 Effect of the 7472insC mutation on structure and function of tRNA$^{Ser(UCN)}$ (II)

The main molecular phenotype of the 7472insC and the A7445G mutations, thus, is a significant decrease in the steady-state levels of tRNA$^{Ser(UCN)}$ that is reflected in a similar clinical phenotype. The differences in the molecular and clinical phenotypes could be explained by specific molecular effects of the 7472insC mutation on the structure of tRNA$^{Ser(UCN)}$, given that the A7445G mutation lies beyond its 3´- end, whereas np 7472 lies within the tRNA itself. As the next step in my study, I therefore analysed the structure of the tRNA in 7472insC mutant and wild-type cybrid cells.

5.2.1 Effect on tRNA base modifications and secondary/tertiary structure

The primary sequence of tRNA$^{Ser(UCN)}$ purified from a wild-type clone (line 34) was determined by a combination of partial RNase digestion and TLC analysis of 5′-postlabelled nucleotides. Four kinds of modifications (two ψ, T, m^{3}C and either ms^{2}i^{6}A or i^{6}A) at five positions (Fig. 5.5) were found and confirmed to be present in the second control clone (line 43). The mutant tRNA isolated from clone 47 had no detectable changes in the pattern of base modifications.

The structural analysis of tRNA was performed by partial RNase digestion under non-denaturing conditions. No major structural rearrangements were found in the mutant tRNA molecule, although the analysis did not resolve the exact location of the extra G.
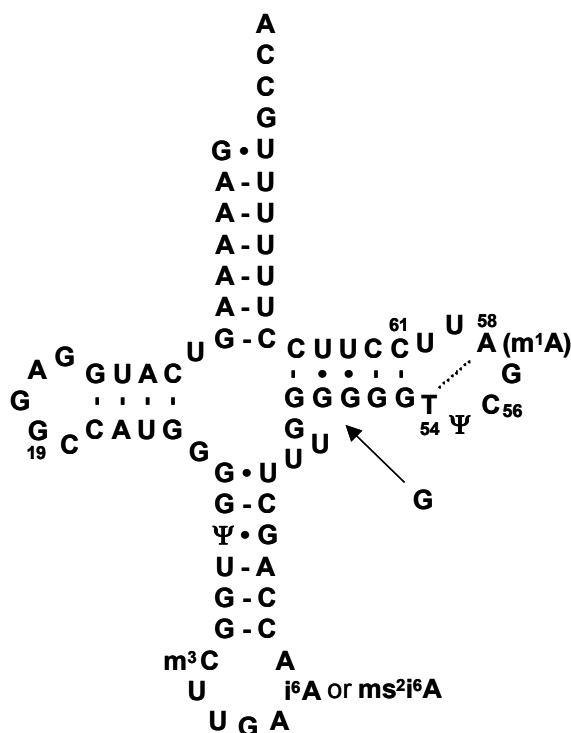

Figure 5.5. *Primary sequence and secondary structure of human mitochondrial tRNA$^{Ser(UCN)}$. Four kinds of base modifications (ψ, pseudouridine; T, ribothymidine; m^{3}C, 3-methylcytidine; i^{6}A, N^6-isopentenyladenosine or ms^{2}i^{6}A, 2-methylthio-N^6-isopentenyladenosine) were identified at five positions by a combination of partial RNase digestion and TLC analysis of single nucleotides (data not shown). In addition, m^{1}A (1-methyladenosine) modification is proposed to be present at position 58 at low frequency based on RT-PCR analysis. Structural analysis (data not shown) did not resolve the exact location of the extra G templated by the 7472insC mutation (indicated by arrow). A tertiary interaction within the reverse Hoogsteen base pair A58-T54 is indicated by dotted line (see Discussion 6.2.1). Numbers indicate conventional positions of nucleotides.*

Sequence analysis of cDNA from natural modified mammalian tRNA[Lys,3], which serves as a primer for HIV reverse transcription, showed that m^{1}A58 is almost always specifically misrecognized by the viral RTase, resulting in the insertion of A instead of T (Auxilien et al, 1999). In my study, RT-PCR on circularized tRNAs using M-MLV RTase produced this misreading of A58 at a low frequency in both wild-type and mutant tRNA[Ser(UCN)] (Table 5.1). The clones were obtained from cells cultured in normal medium or recovered after treatment with low concentration of EtBr (250 ng/ml for 2 days), which did not affect the frequency of misreading. In wild-type clones from cells treated with high doses of EtBr (2.5-5 μg/ml for 4-8h) this frequency was much higher. Most molecules of tRNA[Leu(UUR)], which is known to contain m^{1}A58 (Helm et al, 1999; Yasukawa et al, 2000) were mis-copied at this position. Therefore, this misreading could be used as a marker for the modification. Although high doses of EtBr increased the frequency of misreading, it is unlikely that EtBr itself interferes with the fidelity of reverse transcription. The preferential site of EtBr intercalation into tRNA is suggested to be the bottom of the acceptor stem (Wells and Cantor, 1977; Chu et al, 1997). No other modified or non-modified sites were systematically miscopied by reverse transcriptase in either tRNA. These results suggest that an equally low proportion of wild-type and mutant tRNA[Ser(UCN)] molecules contain m^{1}A58 modification not detected by tRNA sequencing analysis.

Table 5.1. *Misreading of A58 during reverse transcription*

tRNA	Total clones	EtBr	RT error at A58	% of total
Wild-type Ser(UCN)	192 62	No/ low High	3 7	1.6 11.3
np 7472 mutant Ser(UCN)	330	No/ low	5	1.5
Leu(UUR)	111 56	No High	86 52	77 93

5.2.2 Effect on tRNA aminoacylation

The method of oxidation-circularization was chosen, since attempts to separate the aminoacylated and deacylated forms of tRNA[Ser(UCN)], as well as the use of [^{14}C]-serine labelleing prior to analysis on acidic gels were unsuccessful (I). The assay is based on periodate oxidation of tRNA molecules which are not protected by amino acid, whereas the protected tRNA can be circularized after subsequent deacylation and used as a template for RT-PCR amplification. The relative extent of aminoacylation of mutant and wild-type tRNA present in the

same reaction mixture can be then estimated by fluorescent minisequencing. Two minisequencing primers designed for coding (Cser3) and non-coding (Cser4) mtDNA strands gave an average proportion of ~75% of mutant tRNA in the aminoacylated fraction relative to its ratio in total RNA fraction in various arbitrary mixtures (Fig. 5.6). This means that the mutation causes ~25% defect in aminoacylation that would reduce further the steady-state levels of the functional tRNA.
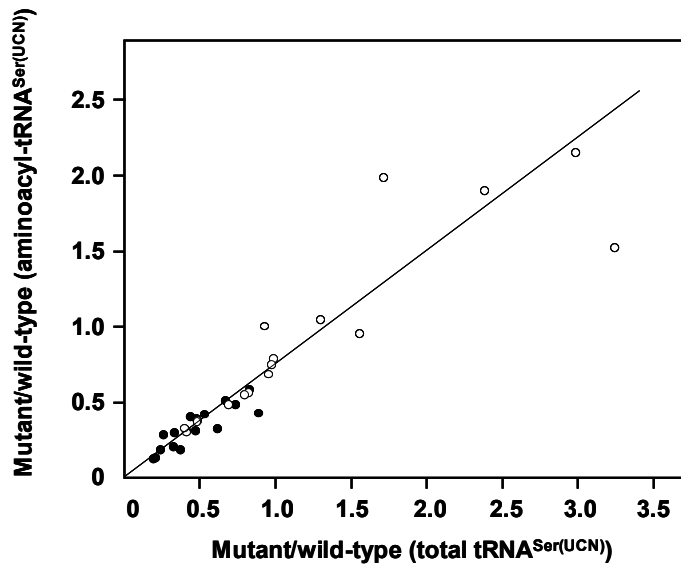

Figure 5.6. *Relative extent of aminoacylation of tRNA^{Ser(UCN)} in control and mutant cybrid clones estimated by the oxidation-circularization assay. Arbitrary mixtures of RNA from control and mutant clones were analysed in two parallel reactions, in one of which tRNAs were deacylated in vitro before the oxidation step. The ratio of mutant and wild-type product in aminoacylated and total (i.e. deacylated in vitro) RNA fractions was determined by RT-PCR and fluorescent minisequencing using primers for coding (open circles) and non-coding (filled circles) strands. The line of slope 0.75 represents an unweighted mean ratio of all data points. Reprinted from original article (II), copyright (2002), by permission of The American Society for Biochemistry and Molecular Biology, Inc.*

5.2.3 Effect on tRNA turnover

The studies on tRNA turnover were performed on cells treated with EtBr to block mitochondrial transcription. The half-lives of tRNA^{Ser(UCN)}, and of tRNA^{Leu(UUR)} used as a reference, were not significantly different in mutant (line 47) and control (line 43) cells (Fig. 5.7a). During EtBr treatment, the relative extent of aminoacylation of the mutant tRNA became slightly decreased (Fig. 5.7b). However, this appeared to have no effect on its turnover.
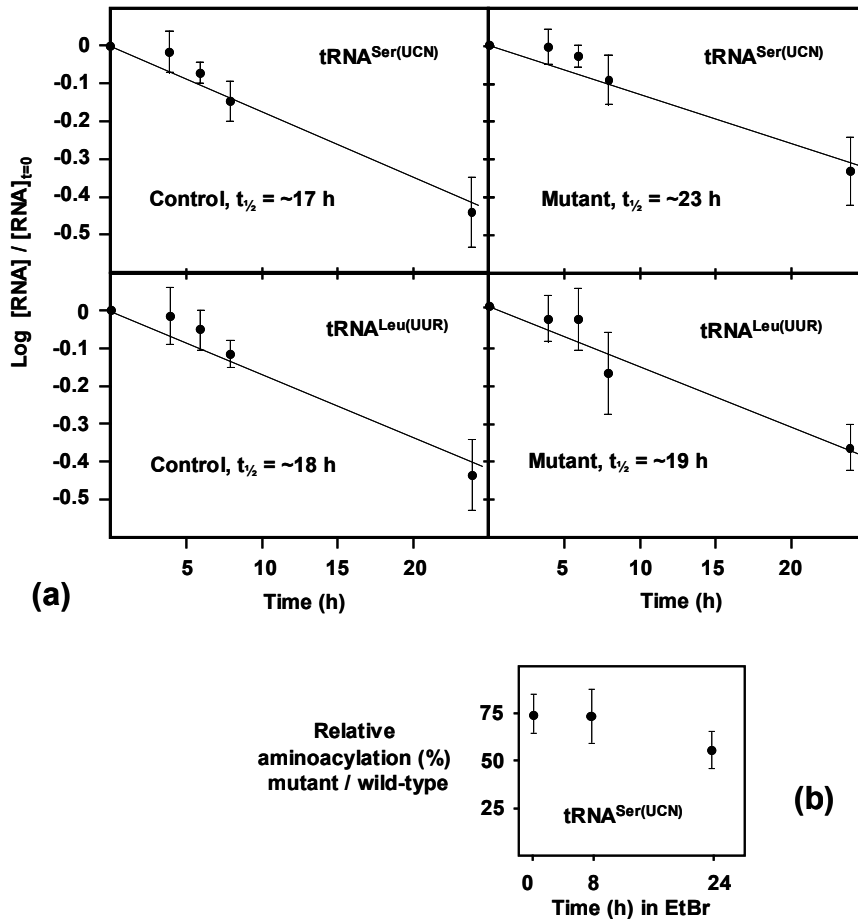
Figure 5.7. *Decay kinetics of mitochondrial tRNAs in control and 7472insC mutant cybrid cells. (a) tRNA levels determined by Northern hybridization with probes specific for tRNA$^{Ser(UCN)}$ and tRNA$^{Leu(UUR)}$ using RNA isolated from cells at different times during EtBr treatment. The signals were normalized against 5S rRNA and expressed as a fraction of the signal at zero time (when EtBr was added). Lines of best fit (least squares method) are shown. (b) Analysis of effects of EtBr treatment on relative aminoacylation of mutant versus wild-type tRNA$^{Ser(UCN)}$, by the oxidation-circularization assay, as in Fig. 5.6. Reprinted from original article (II), copyright (2002), by permission of The American Society for Biochemistry and Molecular Biology, Inc.*

5.2.4 Effect of EF-Tu overexpression

In yeast, overexpression of mitochondrial elongation factor EF-Tu can suppress a number of mitochondrial tRNA mutations causing defects in tRNA processing and function. To test the effect of EF-Tu overexpression on the 7472insC mutation phenotype, mutant (line 47) and wild-type (line 43) cells were transfected with a human EF-Tu expression construct created by J.N. Spelbrink, or with an empty vector used as a control. The expression levels of EF-Tu analysed by Western blotting were moderate, and at least two-fold overexpression had no effect on the mutant tRNA levels (Fig. 5.8).
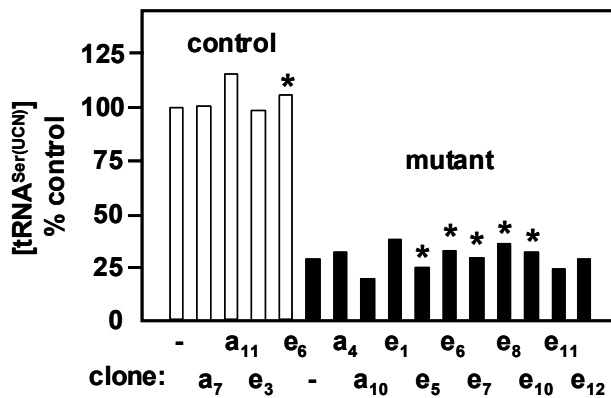
Figure 5.8. *Effect of overexpression of mitochondrial EF-Tu on the steady-state levels of tRNA$^{Ser(UCN)}$ in control and 7472insC mutant cybrid cells. Cells were transfected either with empty vector (clones a) or an EF-Tu expression construct (clones e). The parental cybrid cell lines are denoted (-). Levels of tRNA$^{Ser(UCN)}$ were estimated by Northern hybridization and expressed as a percentage of the signal from the control cell line. Clones with EF-Tu with more than twofold overexpression levels (estimated by Western blotting, data not shown) are denoted by asterisks. Reprinted from original article (II), copyright (2002), by permission of The American Society for Biochemistry and Molecular Biology, Inc.*

5.3 Effect of the 7472insC mutation on the synthesis of tRNA$^{Ser(UCN)}$ (II, III)

Since the 7472insC mutation did not cause a decrease in tRNA stability, the low steady-state levels of mutant tRNA$^{Ser(UCN)}$ were likely to be due to its inefficient synthesis. In the next series of studies, I analysed the molecular effects of the mutation on RNA processing *in vivo* and *in vitro*.

5.3.1 Total rate of tRNA synthesis (II)

tRNA synthesis was studied in cells recovering after prolonged EtBr treatment. The rate of accumulation of newly synthesized mutant tRNA$^{Ser(UCN)}$ was reduced to the extent comparable with the decrease in its steady-state level (Fig. 5.9). Two other L-strand-encoded tRNAs located downstream of tRNA$^{Ser(UCN)}$, tRNA^{Tyr} and tRNA^{Gln}, as well as H-strand-encoded tRNA$^{Leu(UUR)}$ used as a control, were synthesized at rates not different in mutant and wild-type cell lines. Therefore, the 7472insC mutation was deduced to affect synthesis of tRNA$^{Ser(UCN)}$ posttranscriptionally with no polarity effect on other tRNAs of the L-strand transcription unit.
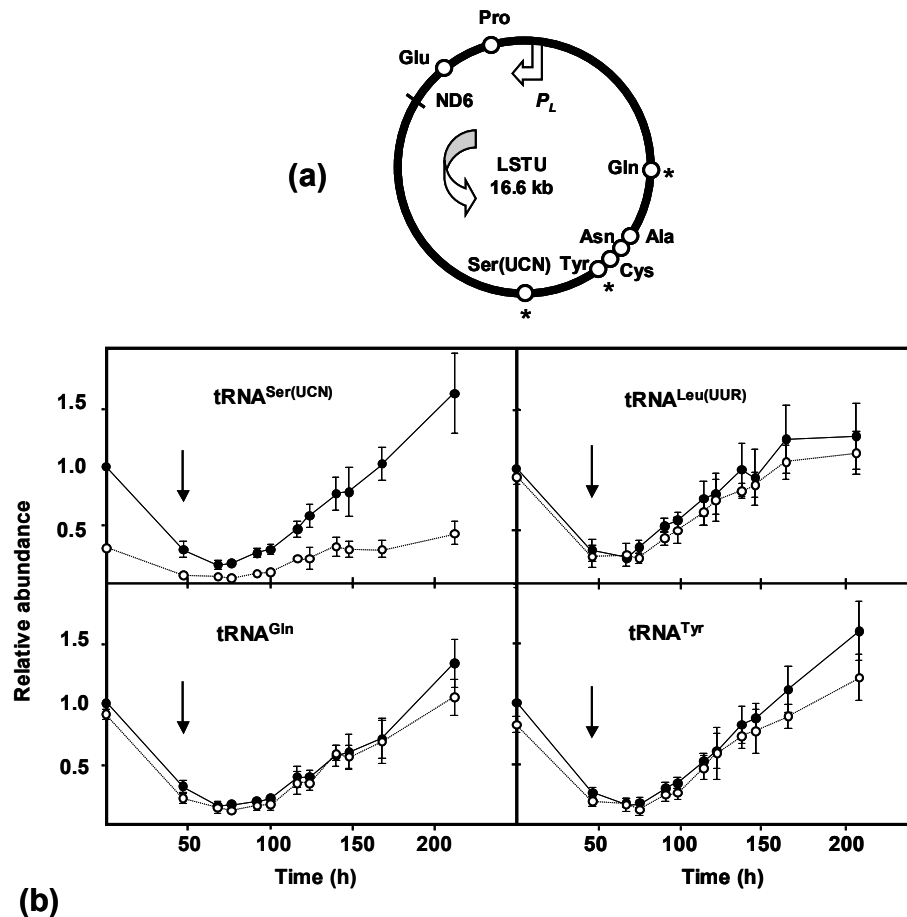
66

Figure 5.9. *Effects of the 7472insC mutation on mitochondrial tRNA synthesis. (a) Schematic diagram of the 16.6-kb LSTU of human mtDNA. Asterisks indicate the light-strand encoded tRNAs whose synthesis was studied in the experiments. (b) tRNA levels during EtBr treatment and recovery were determined by Norhtern hybridization with tRNA-specific probes using RNA isolated from mutant (open circles and dotted lines) and control (filled circles and solid lines) cybrid cells. The signals were normalized against 5S rRNA and expressed as a fraction of the signal for the tRNA in question from control cells at zero time (when EtBr was added). EtBr was removed after 2 days (indicated by arrows) and cells were cultured in fresh medium up to 7 days. Reprinted from original article (II), copyright (2002), by permission of The American Society for Biochemistry and Molecular Biology, Inc.*

Products of the newly synthesised tRNA$^{Ser(UCN)}$(unpublished results)

During studies on tRNA synthesis, a slower migrating band of tRNA$^{Ser(UCN)}$ was observed in both wild-type and mutant RNA fractions (Fig. 5.10). The proportion of this second band, however, was more pronounced in the mutant tRNA. The two bands might represent tRNA isoforms with a difference in base modification. In both cell clones, the accumulation of a presumably undermodified isoform with slower electrophoretic migration was highest at

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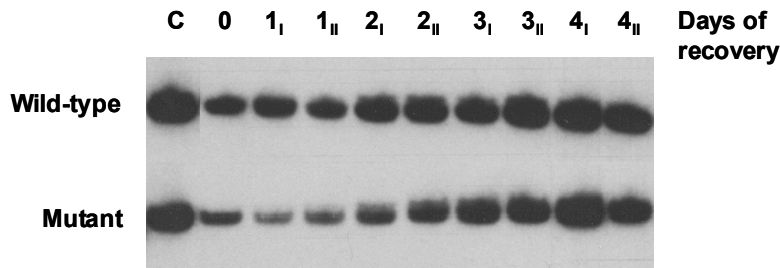early time-points of recovery (days 1 and 2). Such isoforms were not observed for any other tRNAs studied.

Figure 5.10. *Synthesised tRNA$^{Ser(UCN)}$ in the 7472insC cybrid clones following treatment with EtBr. After incubation with EtBr for 2 days (0 days of recovery), cells were cultured in fresh medium. Total RNA was separated on a denaturing gel and hybridized with a probe specific for tRNA$^{Ser(UCN)}$. Samples I and II were collected on the same day with a difference in 8 hours. C, untreated sample.*

5.3.2 Processing of tRNA$^{Ser(UCN)}$ in vivo

The analysis of the products of processing *in vivo* was performed on total RNA isolated from mutant and wild-type cells by circularization, RT-PCR of tRNA$^{Ser(UCN)}$, cloning and sequencing. The mutant tRNA molecules were found to be misprocessed at 5′-, 3′- or both termini with a frequency over 11% (Fig. 5.11 and Table 5.2). The most frequent processing defect was the presence of an extra U nucleotide at the 5′-end that was found uniquely in mutant cells. This defect was often associated with misprocessing at the 3′-end. Both mutant and wild-type cells contained tRNA$^{Ser(UCN)}$ molecules with an incomplete CCA sequence at same frequency, but some of the mutant molecules also had non-templated insertions at the 3′-end.

5.3.3 5′- and 3′-processing in vitro

In vitro RNA processing assays were performed with partially purified HeLa cell RNase P and mitochondrial tRNase Z on pre-tRNA$^{Ser(UCN)}$ constructs containing both 5′-leader and 3′-trailer or 'pre-matured' at one of the termini (Fig. 5.12). The constructs with the 7472insC mutation were processed correctly at the 5′-end. The efficiency of processing in the presence of limiting amounts of RNase P was, however, clearly decreased for the mutant substrate. The relative efficiency of 5′-processing of the mutant substrate was ~60% for several dilutions of the enzyme, although it could not be reliably estimated at very low concentrations.

The 3′-processing efficiency of the mutant substrate was dependent on the presence of the 5′-leader. The construct with mature 5′-end was an efficient substrate for tRNase Z (Fig. 5.13a). In contrast, the construct retaining the 5′-

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leader was very poorly processed even at high concentrations of the enzyme (Fig. 5.13b).
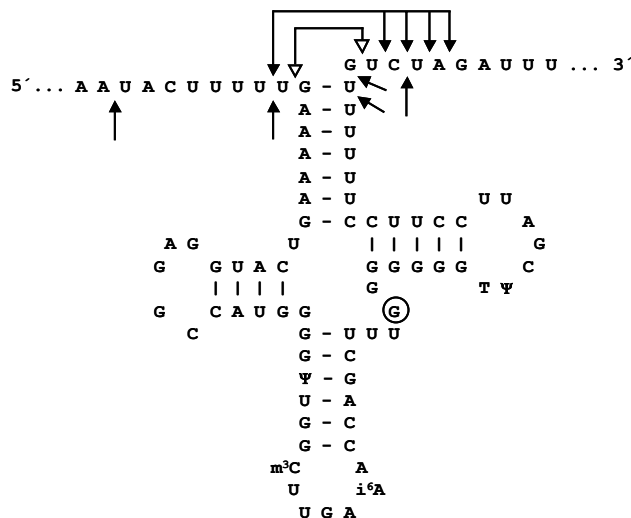

Figure 5.11. *Misprocessed molecules of tRNA^Ser(UCN) detected in vivo in the 7472insC mutant cybrid cell line. The correct processing sites are shown by open arrowheads, the incorrect processing sites are shown by filled arrowheads. Incorrect 3′-processing sites detected in combination with an additional 5′-terminal U are indicated by horizontal line. The additional G templated by the mutation is marked by a circle. Reprinted from original article (III), copyright (2004), by permission of Elsevier.*

Table 5.2. *tRNA^Ser(UCN) molecules with various termini detected in vivo[a]. Reprinted from original article (III), copyright (2004), by permission of Elsevier.*

Class	Wild-type cells	7472insC mutant cells
Total number of clones analysed	192	330
Correct termini (%)	96.9	86.9
5′ misprocessed (total)	<1[c]	6.7
5′ defect only	0	4.2
5′ and 3′ defects	<1[c]	2.5
3′ misprocessed (total)	<1[c]	6.2
3′ defect ± CCA[b]	<1[c]	4.5
3′ defect + inserted nts ± CCA	0	1.7
CCA defect only[b]	2.1	2.0

[a] Approximately equal numbers of clones were analysed from cells grown in normal medium and from cells treated transiently with EtBr, both from wild-type (96+96) and mutant cells (158+172). No significant differences in the frequency of misprocessed forms of tRNA^Ser(UCN) were found under the two conditions, therefore data are pooled.

[b] CCA wholly or partly missing.

[c] Based on single cases.

Figure 5.12. *5´-processing of pre-tRNA$^{Ser(UCN)}$ in vitro. (a) Uniformly labelled wild-type and 7472insC mutant substrates with an 83 nt 5´-leader and 3´-matured end including CCA, 5´-processed to completion with (+) or without (-) excess HeLa cell RNase P. (b) Uniformly labelled wild-type and 7472insC mutant substrates with an 83 nt 5´-leader and a 25 nt 3´-trailer, 5´-processed to completion with (+) or without (-) excess RNase P. (c) The same substrates as in (b), wild-type (w) and 7472insC mutant (m), 5´-processed using increasing amounts (arbitrary units) of RNase P. (d) Relative efficiency of the same substrates as in (b, c) using increasing amounts of RNase P. The mutant and wild-type substrates mixed in equal proportions were 5´-processed and the ratio of gel-extracted products to remaining substrates, normalized against unprocessed substrate, was estimated by RT-PCR and minisequencing. The overall extent of reaction was determined densitometrically, in each case, from the ratio of products to substrate. The dotted line indicates that the mutant substrate was processed at the relative efficiency of ~60% for several dilutions of the enzyme. Reprinted from original article (III), copyright (2004), by permission of Elsevier.*
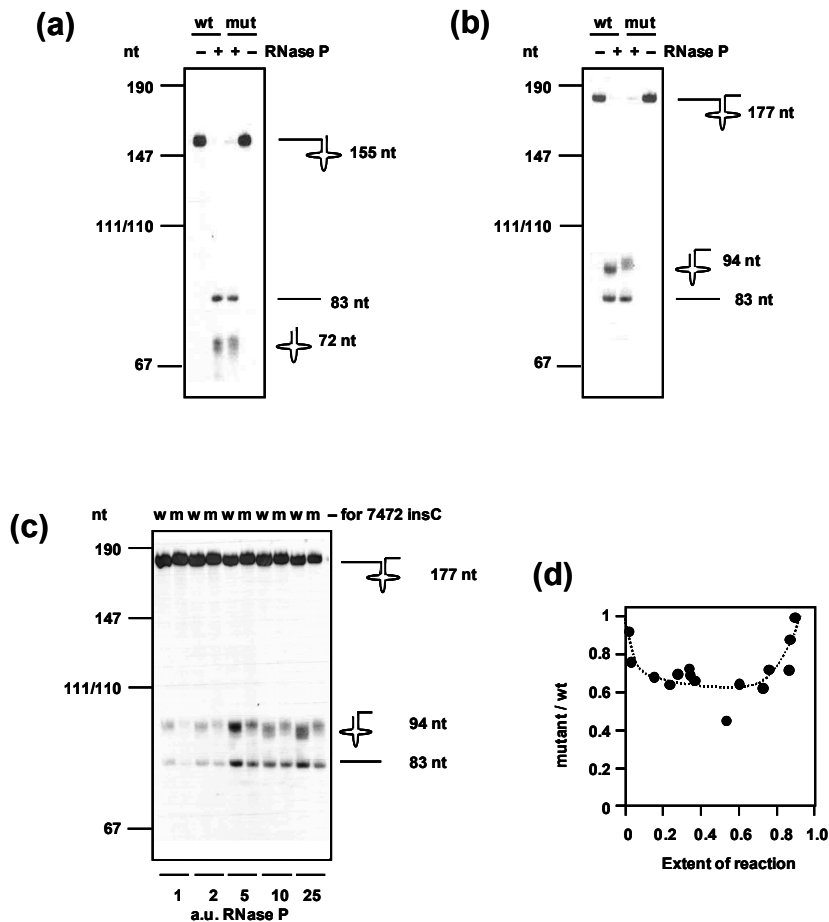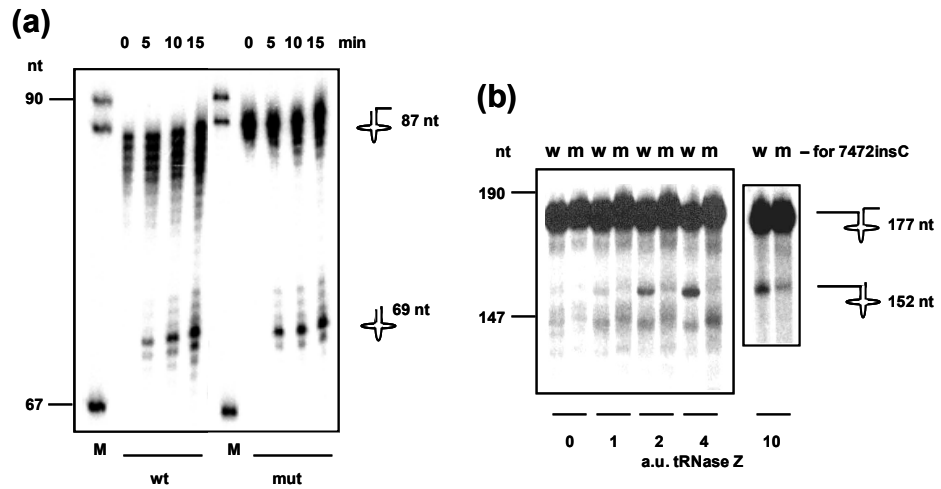
Figure 5.13. *3′-processing of pre-tRNA$^{Ser(UCN)}$ in vitro. (a) 5′-end labelled wild-type and 7472insC mutant substrates with an 18 nt 3′-trailer and 5′-matured end, 3′-processed for increasing times with partially purified mitochondrial tRNase Z from HeLa cells. (b) Uniformly labelled wild-type (w) and 7472insC mutant (m) substrates with an 83 nt 5′-leader and a 25 nt 3′-trailer, 3′-processed using increasing amounts (arbitrary units) of tRNase Z. Reprinted from original article (III), copyright (2004), by permission of Elsevier.*
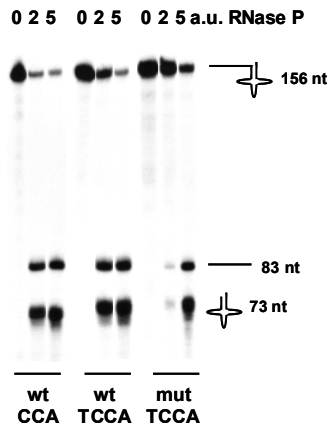
Figure 5.14. *5′-processing of 3′-misprocessed pre-tRNA$^{Ser(UCN)}$ in vitro. Uniformly labelled wild-type and 7472insC mutant substrates with an 83 nt 5′-leader, plus the 3′-misprocessed end (discriminator G)-UCCA or the correct 3′-end (discriminator G)-CCA, 5′-processed using increasing amounts (arbitrary units) of RNase P. Reprinted from original article (III), copyright (2004), by permission of Elsevier.*

5.3.4 Interference of 3′-misprocessing with 5′-processing

A possible interference of 3′-misprocessing with 5′-processing was tested on substrates that contained an additional 3′-U nucleotide before the CCA end. The mutant construct was processed by RNase P less efficiently than the wild-type

(Fig. 5.14), and there was some evidence of 5′-misprocessing occurring in the mutant substrate.

5.4 Polyadenylation of human mitochondrial tRNAs (unpublished results)

In this chapter, I provide some results of studies relevant to the tRNA turnover mechanism in human mitochondria. In bacteria and yeast, defective tRNAs are degraded through polyadenylation (Li Z et al, 2002; Kadaba et al, 2004). My results suggest that a similar mechanism of tRNA quality control might also exist in human mitochondria.

5.4.1 Effect of high doses of EtBr on human mitochondrial tRNAs

These results originate from my observation that, in cells treated with high doses of EtBr (1-10 μg/ml for 4-24 h), a proportion of the mitochondrial tRNA molecules are modified, migrating electrophoretically as higher molecular weight products up to 200 nt in length (Fig. 5.15).
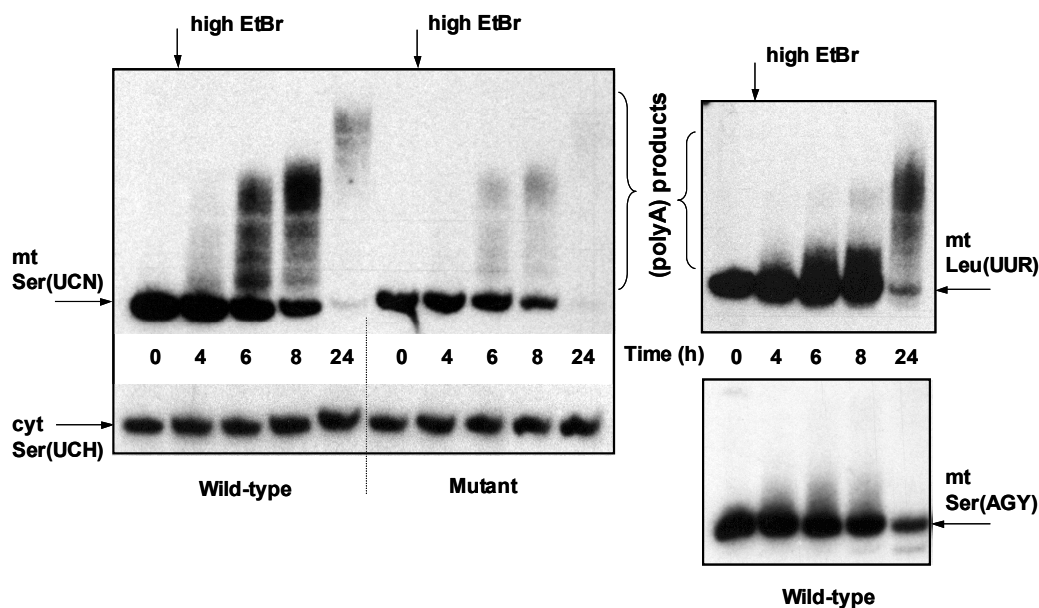

Figure 5.15. *Effect of high doses of EtBr on human mitochondrial tRNAs. Total RNA from wild-type and 7472insC cybrid cells treated with 2.5 μg/ml of EtBr for the indicated times was hybridized with tRNA-specific probes. The high molecular weight products were confirmed to contain poly(A) tails at their 3′-ends (see the text and Table 5.3).*

Table 5.3. *Cloning of EtBr-induced high molecular weight tRNA products*

tRNA	Ser(UCN)		Leu(UUR)	
EtBr, µg/ml	3.5	5	5	5
Time of treatment, h	4; 6	4; 6	8	24
Total number of clones	38	20	41	15
Intact tRNA	30	10	3	1
Intact tRNA + poly(A)	4	5	7	6
Examples of poly(A) sequences	$A_{(1)}$; $A_{(4)}$ $T_{(1)}$ $A_{(3)}CA_{(6)}$	$A_{(7)}$; AAC $A_{(8)}CA_{(8)}$ $A_{(38)}TTA_{(21)}$ $A_{(25)}TA_{(28)}C$	$A_{(1-2)}$; $A_{(4)}$ TA	$A_{(1-3)}$; $A_{(5-6)}$
Truncated tRNA	3	4	0	0
Truncated tRNA + poly(A)	1	1	31	8
Examples of poly(A) sequences	$A_{(14)}$	$A_{(6)}$	$A_{(2-6)}$; $A_{(8-9)}$ $A_{(13)}$; GA CAA; $TA_{(5)}$ $AACA_{(2/5)}$ AGCAA	$A_{(7)}$; $A_{(9)}$ $A_{(11-12)}$ $A_{(14)}$ $A_{(25)}CA_{(12)}$ $A_{(14)}GA_{(4)}$-$C_{(12)}$
% (non-A) total	4.85		3.67	

To identify the nature of these tRNA-related products in EtBr-treated cells, total RNA from wild-type cybrid cells was isolated. tRNAs were deacylated, cirularized, RT-PCR-amplified, cloned and sequenced. This analysis was performed for tRNA[Ser(UCN)] and tRNA[Leu(UUR)]. The high molecular weight products were found to be either intact or truncated tRNA molecules containing poly(A) homo- or heteropolymers at their 3′-ends (Table 5.3). The internally polyadenylated tRNA molecules lacked the complete CCA sequence at their 3′-ends, most often missing the CA terminus, or were truncated further to various extents (missing 3-14 nucleotides of the encoded tRNA sequence), up to the T-loop. Heteropolymeric tails contained a low proportion (4-5%) of non-A residues (C, G or T). The frequency of such incorporations, however, cannot be reliably estimated from these results, due to a preferential amplification of short tRNA molecules with oligo(A) tails. The identified products rather represent qualitative data.

The effect of EtBr was specific to mitochondrial tRNAs. Cytosolic tRNA[Ser(UCH)] (see footnote[1]), as well as 5S rRNA (data not shown) were not affected by EtBr treatment. The extent of polyadenylation increased in proportion to the applied dose of EtBr and the time of exposure. The effect

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[1] This tRNA is expected to read the codons containing A, C or U (denoted as H) at the wobble position, due to the post-transcriptional conversion of the adenosine encoded at the first position of the anticodon (Krupp et al, 1988) to inosine.

varied, however, between different mitochondrial tRNAs. Out of eight tRNAs tested (Table 5.4), tRNA^{Phe} was the most sensitive to EtBr treatment, whereas tRNA$^{Ser(AGY)}$ became only partially oligoadenylated and, at higher doses of EtBr, polyadenylated to a minimal extent, which was accompanied by tRNA degradation. For wild-type as well as the 7472insC mutant tRNA$^{Ser(UCN)}$, the effect was similar.

Table 5.4. *The extent of polyadenylation of mitochondrial tRNAs. tRNAs are classified based on inspection of Northern hybridization signals.*

low						high
	tRNA$^{Ser(AGY)}$ tRNA^{His}	tRNA$^{Leu(UUR)}$	tRNA^{Lys}	tRNA$^{Ser(UCN)}$ tRNA^{Gln} tRNA^{Trp}	tRNA^{Phe}	

5.4.2 Turnover of polyadenylated tRNAs

Presumably because of aberrant structure, resulting from the presence of the drug (see Discussion 6.4.1), mitochondrial tRNAs therefore appear to become polyadenylated. To test the stability of these products, cells were incubated in fresh medium following EtBr treatment. After removal of the drug, polyadenylated tRNAs were rapidly degraded, with a half-life of ~1.5 h (Fig. 5.16). The degradation was accompanied by a gradual recovery of tRNA levels. These results suggest that mitochondrial tRNA molecules with an aberrant structure, which does not directly lead to tRNA instability, are turned over following polyadenylation, but only in the absence of EtBr.
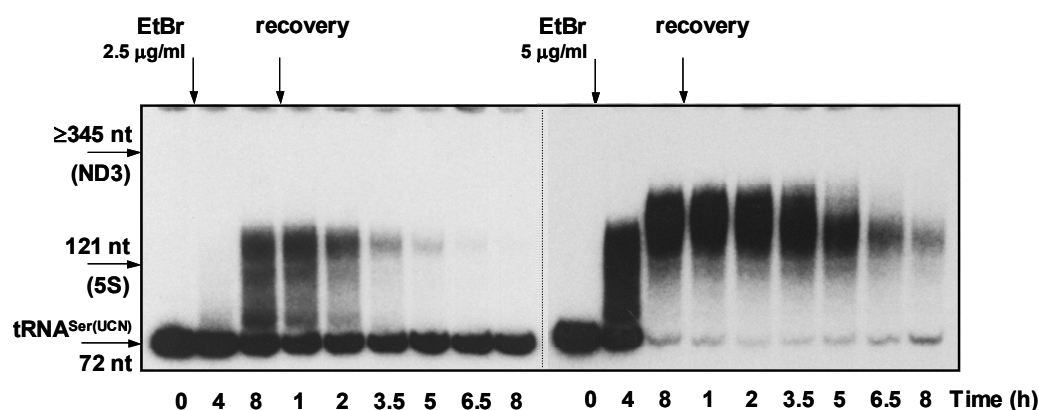

Figure 5.16. *Turnover of polyadenylated tRNAs. Cells were treated with EtBr followed by a period of recovery in fresh medium. Total RNA was hybridized with probes specific for tRNA$^{Ser(UCN)}$ and tRNA$^{Leu(UUR)}$ (not shown). Positions of ND3 mRNA and 5S rRNA are provided as length markers.*

Table 5.5. *Effect of inhibitors of the OXPHOS system and mitochondrial translation on EtBr-induced tRNA polyadenylation.*

Drug	Inhibition site	Effect on tRNA polyadenylation
Oligomycin	ATP synthase	Dose-dependent inhibition
CCCP	Uncoupling of respiratory chain	Strong inhibition
Doxycycline	tRNA binding to mt ribosome	Strong inhibition
Thiamphenicol	Elongation of mt translation	Transient inhibition
Puromycin	Transpeptidation	Partial inhibition
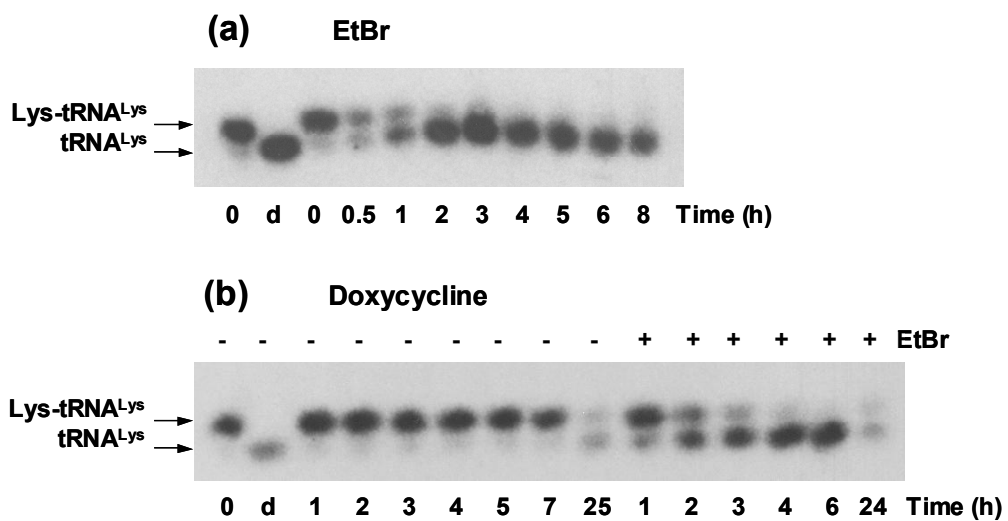
Figure 5.17. *Effect of EtBr and doxycycline on mitochondrial tRNA aminoacylation. The example of tRNA^{Lys} is provided, for which charged and non-charged forms can easily be separated by acidic PAGE. (a) Cells were treated with 5 μg/ml EtBr for the indicated times. Total RNA was isolated, separated under acidic conditions and hybridized with a tRNA^{Lys}-specific probe. d, deacylated sample prepared by heating for 10 min at 75 °C followed by 30 min at 37 °C in 1.5 volumes of 0.5 M Tris-HCl pH 9.0. (b) Cells were treated with 200 μg/ml doxycycline either alone or in combination with 5 μg/ml EtBr added after 1 h of preincubation with doxyxycline (not included in the indicated time). Total RNA was analysed as in (a).*

5.4.3 Studies on the mechanism of tRNA polyadenylation

In order to investigate further the mechanism of tRNA polyadenylation, I performed various treatments of cells with inhibitors of the respiratory chain and mitochondrial protein synthesis, either alone or in combination with EtBr (Table 5.5). Full results of these studies will be communicated in a future publication. To summarize, tRNA polyadenylation appeared to be dependent on both ATP availability and on active mitochondrial translation. Blocking of translation (e.g.

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with doxycycline) inhibited also the turnover of polyadenylated products. EtBr induced the rapid deacylation of tRNA, which was delayed by inhibitors of mitochondrial protein synthesis (Fig. 5.17). However, the correlation between the aminoacylation state of individual tRNAs and the extent of polyadenylation was not precise, suggesting that prior deacylation is necessary but not sufficient for polyadenylation to occur. My results suggest that mitochondrial tRNA polyadenylation and turnover may be associated with the ribosome.

6. DISCUSSION

6.1 Molecular phenotype of the 7472insC mutation (I)

The aim of the first part of my work was to characterize the molecular phenotype of the 7472insC mutation in patient-derived osteosarcoma cybrid cells, and to compare it to the previously described pathogenic mutation affecting tRNA$^{Ser(UCN)}$ at np 7445 (Reid et al, 1994). Both mutations are mainly deafness-associated. However, they exhibit different accompanying clinical features (Tiranti et al, 1995; Sevior et al, 1998; Verhoeven et al, 1999). The major effect of both mutations in cultured cells was a >60% drop in the steady-state levels of tRNA$^{Ser(UCN)}$. This did not, however, affect to a significant extent the ability of cells to grow under respiratory stress, and did not detectably impair mitochondrial protein synthesis.

Although these results relate the deafness-associated mutations to a deficiency of tRNA$^{Ser(UCN)}$, the connection between the clinical and molecular phenotypes remains unclear. The reasons for such a relationship are also currently unknown for any other tRNA mutation. A highly variable phenotype caused by a single mutation is a common feature of many pathological tRNA mutations, and it is not well understood either. However, the complexity of the clinical features suggests that several genetic and environmental factors could modulate the expression of the disease.

6.1.1 Tissue specificity

A deficiency in overall mitochondrial protein synthesis could be a common feature of deafness-associated mutations. The question of tissue specificity, however, remains unexplained. Signal transduction in cochlear cells is highly dependent on the ionic environment. A number of mutations in 'non-syndromic deafness genes' affect the ion transport system, in particular K^+ recycling (reviewed by Ikeda, 2004). A reduced rate of ATP production may change the ionic balance in cochlear cells that, in combination with other specific factors, predisposes to deafness.

A deficiency of tRNA$^{Ser(UCN)}$ could specifically affect cochlear cells, since most of the mutations in this tRNA are associated primarily with sensorineural deafness. At least for some of them, such as the A7445G mutation (Reid et al, 1997; Guan et al, 1998), the T7511C mutation (Li et al, 2004), the T7512C and

G7497A mutations (Möllers et al, 2005), and, in my study, the 7472insC mutation, the steady-state levels of the mutant tRNA are demonstrated to be decreased. The translation defects caused by these mutations in cybrid cells were rather quantitative. In accordance with that, analysis of the usage of UCN codons indicates their wide distribution in all protein-coding genes (Jacobs, 2003). However, the polypeptides with conserved Ser(UCN) codons tend to be the most affected, based on *in vitro* translation studies using mitochondria from cells of patients with the 7472insC mutation (Pulkes et al, 2005). Strikingly, despite the extremely low steady-state levels of the tRNA$^{Ser(UCN)}$ with the T7512C and G7497A mutations, the decrease in protein synthesis was comparable to that in the case of the A7445G mutation (Möllers et al, 2005; Guan et al, 1998).

A threshold of ~40% of the usual level of tRNA$^{Ser(UCN)}$ is proposed to be required for a normal rate of translation (Guan et al, 1998). Since the levels of tRNA$^{Ser(UCN)}$ with the 7472insC and the A7445G mutations are close to this value, any small change in abundance or quality of multiple factors involved in translation may produce a combination with a sharp tissue-specific effect. The abundance of tRNA$^{Ser(UCN)}$ seems to vary between different tissues (Reid et al, 1997). In addition, the mutant tRNA itself may be less efficient in translation, so that the proportion of wild-type tRNA in heteroplasmic patients could also be critical. In mouse, the expression of mt SerRS was found to be low in brain and skeletal muscle (Gibbons et al, 2004). Based on PCR analysis, mt SerRS is expressed in the inner ear, although its relative levels remain to be estimated (Gibbons et al, 2004).

6.1.2 Modifying factors

One of the modifying factors suggested by my studies could be the copy number of mtDNA or anything which modulates it, as a low copy number correlated with a cell growth disadvantage under the respiratory stress. Previously, higher copy numbers of mutant mtDNA were shown to improve the respiration of osteosarcoma cybrids with the A3243G mutation (Bentlage and Attardi, 1996). On the other hand, this may reflect a consequence rather than a modifier of the primary mutation. The functional defect of mutant mitochondria may produce more oxidative damage to the cell due to ROS generation. Elevated oxidative stress may serve as a signal for an increase in mtDNA copy number that, in turn, would cause further increase in ROS as a vicious cycle (reviewed by Lee and Wei, 2005). The regulation of mtDNA copy number is not well understood, but such factors as TFAM (Ekstrand et al, 2004) and Twinkle (Tyynismaa et al, 2004) have been proposed to be essential. These and other regulatory factors could contribute to the variability in mtDNA copy numbers between different tissues and in individuals. In skeletal muscle, contractile activity is known to stimulate mitochondrial biogenesis that leads to upregulation of TFAM and increase in mtDNA copy number (reviewed by Hood, 2001). For patients with heteroplasmic mitochondrial myopathies, however, a benefit of

exercise training is uncertain, as endurance training may selectively increase proliferation of the mutant mtDNA (reviewed by Taivassalo and Haller, 2004). In the case of sporadic mutations, resistance training is suggested as a possibility to increase the proportion of wild-type DNA by activation of satellite cells.

My study might support also a possibility that some drugs, in particular antibiotics, could play a role as environmental cofactors, with an effect on mitochondrial function. Thus, in my experiments, mitochondrial translation in a mutant cell line showed a mild hypersensitivity to doxycycline, although a high concentration of the drug was required to see the effect. A hypersensitivity to doxycycline was observed also for the *tko* mutant *Drosophila* that carries a mutation in the mitochondrial ribosomal protein S12, causing hearing impairment and other mitochondrial disease-like phenotypes (Toivonen et al, 2001). Since mitochondrial ribosomes are bacterial-type, they may be sensitive to antibiotics that inhibit bacterial translation. The ototoxic effect of aminoglycosides in combination with the A1555G mutation in the 12S rRNA gene has been well established (Fischel-Ghodsian, 2003). Tetracycline, which blocks tRNA binding to the ribosome, presumably in the same way as its analogue doxycycline, has been shown to inhibit mitochondrial and bacterial translation with a similar efficiency *in vitro* (Zhang et al, 2005). Although inside the cell, mitochondrial permeability probably has a protective role, the limited functional capacity of the mitochondrial translational machinery in mutant cells might be potentially more sensitive to any such effects.

In patients, genetic factors such as additional mutations or polymorphisms could also act as modifiers of mitochondrial function. For example, variable penetrance of deafness between two pedigrees with the T7511C mutation is suggested to be due to the presence of additional mutations in the ND1 gene and in the gene for tRNA[Ala] in one of the families (Li et al, 2004; Li R et al, 2005). The mutations result in a significant decrease in the steady-state levels of tRNA[Ala], ND1 mRNA and the adjacent tRNA[Leu(UUR)]. However, both mutations may be neutral polymorphisms in some populations. The additional A7472C transition is speculated to have a suppressive effect on the 7472insC mutation, since it was found to be associated with a milder disease phenotype (Pulkes et al, 2005; Cardaioli et al, 2006). Although in cultured human cells mitochondria were observed to fuse at a very low frequency, potentially, some mtDNA mutations might undergo transcomplementation or result in additive effects (Enriquez et al, 2000). Recent studies on skeletal muscle of several individuals suggest that mtDNA recombination could be a common event (Zsurka et al, 2005). Cases of late onset of the disease and general progression of symptoms with age could thus be considered to support the notion of age-dependent accumulation of modifying (worsening) mitochondrial mutations.

6.1.3 Cybrid model

Cybrid cell technology provides a powerful method for studying mitochondrial tRNA mutations. The main advantage of this technique is that it allows to achieve homoplasmy or different loads of the mutation and to vary the nuclear background. Alternative models could be the primary cell cultures or biopsies from patients, which provide, nevertheless, a rather restricted amount of material. A drawback of the *S. cerevisiae* model is the considerable amount of structural difference between yeast and mammalian mitochondrial tRNAs (Florentz et al, 2003). For functional studies, *in vitro* tRNA transcripts have been widely used, although the lack of base modifications should be taken in account.

The cybrid model also has its own limitations. Firstly, as discussed above, the disease may be restricted to a specific tissue, such as cochlear cells in the case of sensorineural deafness. Therefore, the cellular background of the model may not correspond to the special metabolic and energetic requirements of the affected tissue. The cybrid cells used in my work were initially derived from an osteosarcoma cell-line with mitochondria obtained from a patient's myoblasts with the 7472insC mutation (Tiranti et al, 1995). Biochemically, these clones showed a mild decrease in oxygen consumption and complex I activity in comparison with control cybrids, whereas in the donor patient's muscle biopsy both complex I and IV activities were reduced to a greater extent.

The biochemical threshold effect of the respiratory chain complexes is proposed to contribute to tissue specificity (Rossignol et al, 1999). It implies that different tissues have different sensitivities to a defect in a respiratory chain complex, depending on the threshold value for that complex and on the steady-state rate of respiration. In human, brain tissue is predicted to be most sensitive to complex I deficiency, whereas in skeletal muscle COX activity may be critical (Kunz, 2003). The activity of COX may be controlled by tissue-specific isoforms. Three of the nuclear COX subunits (VIa, VIIa and VIII) are represented in muscle-specific (H) and non-muscle-specific (L) isoforms (Grossman and Lomax, 1997). Complex I has been shown to exist in slowly interconverting active-inactive forms that might be involved in regulation of its activity (Grivennikova et al, 2001).

The nuclear background of the recipient cybrid cell line may contribute to the variability also in the molecular phenotype. In studies on the A3243G mutation, differences in the abundance and aminoacylation of tRNA$^{Leu(UUR)}$ and in mitochondrial translation products were observed in different cell lines (Jacobs and Holt, 2000). The suppressive effect of the G12300A mutation in tRNA$^{Leu(CUN)}$ was also dependent on the nuclear background (Lehtinen, 2001). On the other hand, in two different studies of the A7445G mutation in lymphoblastoid cells (i.e. not cybrids) carrying mitochondria derived from different patients, a defect in mitochondrial translation was observed only in one family (Reid et al, 1997; Guan et al, 1998). Therefore, a similar nuclear background may still result in different phenotypes that could possibly be applied even to patient's own cells. In a further study in osteosarcoma cybrids

with mitochondria transferred from lymphoblastoid cell lines, the mutation produced a milder decrease in the steady-state levels of tRNA$^{Ser(UCN)}$ (Li X et al, 2005).

Secondly, tumor-derived cells tend to accumulate chromosomal alterations that may affect mitochondrial function. Thus, trisomy-9 in cybrid cells was found to be associated with transient heteroplasmic instability of the G12300A suppressor mutation (Lehtinen et al, 1999). Chromosomal rearrangements were suggested to be responsible for galactose-resistant phenotype of cybrids with the G5703A mutation in tRNA^{Asn} acquired at high frequency in different nuclear environments (Hao et al, 1999). Recovery of respiratory competence was accompanied by >2-fold increase in the steady-state levels of tRNA^{Asn}. In my work, one of the mutant clones studied also had a notably higher steady-state level of tRNA$^{Ser(UCN)}$ and ND6 mRNA, which I was unable to relate to any sequence differences in mtDNA.

In summary, the cybrid cell model can help to understand the mutation phenotype only partially. Biochemical studies could be completed by different methods of respiratory activity analysis, for example, by inhibitor titration of the individual respiratory chain complexes (Villani and Attardi, 1997). Cybrid cells with different combinations of nuclear background and mitochondrial origin would contribute further understanding of the roles of such factors in the mutant phenotype. Analysis of tissue-specific expression of the relevant genes may also help, in part, to explain the clinical phenotype of the mutation. A disease-specific decrease in expression of a set genes involved in oxidative phosphorylation has been found in association with diabetes (Mootha et al, 2003a). Application of proteomics could be useful in search for tissue-specific differences in mitochondrial protein composition, as demonstrated in several mouse tissues (Mootha et al, 2003b).

Finally, in any such study, multiple independent clones should be analysed in order to avoid any possible artefacts. Although cybrid clones used in my work were derived from one patient, several of them appeared to be 'exceptional', e.g. in carrying an additional insertion at np 7472 (see below), by manifesting higher levels of tRNA$^{Ser(UCN)}$ and also exhibiting a retarded growth rate under respiratory stress. Therefore, the molecular phenotype of these clones should be deduced rather from the average.

6.1.4 Origin of the 7472insC mutation

The 7472insC mutation lies within a stretch of six Cs that is likely to be predisposed to replication slippage. In my study, one of the cell clones was found to have an additional (heteroplasmic) C insertion within the tract at np 7472, which might account for a further destabilization of this sequence by the 7472insC mutation. An additional such insertion was not observed in patients, but could easily be missed by SNP analysis, or due to a low level of heteroplasmy. However, an additional A to C transition at the np 7472 has

recently been found in patients in a peculiar association with the 7472insC mutation (Pulkes et al, 2005; Cardaioli et al, 2006).

In the D-loop region, sequences of more than six consecutive cytosines are often associated with length variation as a result of replication slippage, which could be influenced by mtDNA polymorphisms and by nuclear factors (Malik et al, 2002). Remarkably, in the D-loop region of mtDNA of two patients with the 7472insC mutation the proportion of molecules with these C-tracts of length over six residues was >90%, versus only 10% in control (Pulkes et al, 2005). The exact mechanism predisposing oligo(C) tracts to replication errors is not yet explained. A common mechanism may underlie their occurrence. The origins of the 7472insC mutation were found to be independent in six families from Western Europe (Hutchin et al, 2001).

Some features of mtDNA replication may influence also the segregation of the 7472insC mutation. A replicative advantage of mutant mtDNA is suggested to explain rapid segregation towards homoplasmy in a patient whose mother was only 4% heteroplasmic for the mutation (Schuelke et al, 1998), as well as in initially heteroplasmic patient-derived cybrid cell clones (Tiranti et al, 1995). Such a mechanism might also be responsible for a shift towards the mutant mtDNA in cultured cells (Yoneda et al, 1992). Analysis of germline transmission of several heteroplasmic mutations supports a weak selection effect (Chinnery et al, 2000). This could, however, equally be explained away as sampling or ascertainment bias. Distribution of the A7472C transition in a pedigree did not correlate with the heteroplasmy level of the 7472insC mutation, which suggests that the segregation of the two mutant mtDNA types is independent (Cardaioli et al, 2006).

6.2 Effects of the 7472insC mutation on molecular function of tRNA$^{Ser(UCN)}$ (II)

One obvious difference between the 7472insC and the A7445G mutations is that the latter is located beyond the 3′-end of tRNA $^{Ser(UCN)}$, so that it was expected and later confirmed to affect tRNA processing (Levinger et al, 2001; Yan H et al, 2006). The potential mechanisms of the 7472insC mutation could be multiple, including tRNA structure, function and synthesis. My analysis showed that the mutation had no major effect on tRNA structure, but caused a 25% decrease in aminoacylation. I found that the low steady-state levels of the mutant tRNA$^{Ser(UCN)}$ were not due to a decrease in stability (half-life) and were not complemented by EF-Tu overexpression.

6.2.1 Effect on tRNA structure

The 7472insC mutation did not cause any significant structural changes in tRNA$^{Ser(UCN)}$ that could be detected in our system. Some minor differences, however, could be noted. First, the cytidines in the T-arm were slightly less accessible for enzymatic cleavage under non-denaturing conditions. C56 (conventional positions indicated) forms a conserved tertiary pair with G19 that locks the D- and T-loops at the corner of the L-form (Watanabe at al, 1994; Dirheimer at al, 1995). C61 flanks on one side the bulged dinucleotide 59-60 that is suggested to be essential for maintenance of the L-form (Steinberg et al, 1997; Zagryadskaya et al, 2003). Although the exact location of the additional G in the tertiary structure remains uncertain, the observed differences might support a suggestion that the extra G forms a pair with U60 (Tiranti et al 1995), and thus do not exclude an effect on tRNA tertiary structure.

Second, although no change in the primary sequence of the tRNA was observed, the mutation might still affect the *efficiency* of base modifications (see also the next chapter). In support of that, a slower migrating band that appeared in the newly synthesised tRNA$^{Ser(UCN)}$ after blocking of transcription by EtBr was more pronounced in the mutant RNA fraction (our unpublished observation, see Results 5.3.1 and Fig. 5.10). Several isoforms of tRNA$^{Ser(UCN)}$ were observed both in 2D gel fractionation (King and Attardi, 1993) and in high-resolution denaturing gels where their ratio varied between cell types (El Meziane et al, 1998a). It is highly likely that these isoforms represent tRNAs with differences in base modifications. A similar, presumably undermodified tRNA$^{Ser(UCN)}$ species accumulated during early recovery after EtBr treatment, as observed by Möllers et al (2005). A difference in gel migration may be caused by alternative structures of tRNA, which can be stabilized by base modifications (Helm et al, 1998; Sohm et al, 2003; Helm et al, 2004). A slower migration of tRNA in denaturing gels in my work might be due to a difference in charge that could also be caused by base modifications. Thus, the maturation of the 7472insC mutant tRNA$^{Ser(UCN)}$ might be less efficient.

Third, the levels of base modifications vary depending on the developmental and metabolic state of the cell and may have a regulatory effect on the activity of tRNA in translation (Björk, 1995). Such a transient or partial modification in tRNA$^{Ser(UCN)}$ might be present at position A58 that was misread as A at a low frequency by reverse transcriptase (see Results 5.2.1). Such misreading was shown to almost always occur in the case of m^{1}A58 modification (Auxilien et al, 1999) and, indeed, in my analysis, was observed in most molecules of tRNA$^{Leu(UUR)}$, which is known to be methylated at this position (Helm et al, 1999; Yasukawa et al, 2000). Although the extent of m^{1}A58 modification of tRNA$^{Ser(UCN)}$ in our system seems to be very low (< 2%) in both mutant and wild-type cells, and cannot be judged reliably by such analysis, potentially it may have important functions that could be expressed in different cellular background or under different conditions. These results do not, however, provide any evidence of interference of the 7472insC mutation with the m^{1}A58 modification,

although such an effect might be speculated upon, based on local rearrangement of the T-loop.

A highly conserved reverse Hoogsteen pair A58-T54 is proposed to play a central role in tRNA function by preserving the conformation of dinucleotide 59-60, which determines the general geometry of the L-form (Zagryadskaya et al, 2003). The stability of the A58-T54 interaction is strongly increased by m^{1}A58 modification (Oliva et al, 2006). In yeast, the m^{1}A58 modification is critical for the stability of initiator tRNA^{Met}, which contains A54 instead of T54 (Anderson et al, 1998; Kadaba et al, 2004). In mycobacteria, m^{1}A58 is suggested to stabilize tRNAs that lack the T54 modification (Varshney et al, 2004). Interestingly, bovine mitochondrial tRNA$^{Ser(UCN)}$ also contains unmodified U54 in combination with m^{1}A58 (Watanabe et al, 1994), whereas our results suggest an opposite distribution of modifications in the A58-T54 pair of the human tRNA (Fig. 5.5). Although in these cases the occurrence of the A58-T54 pair modifications seems to be concomitant, the stabilizing effect of T54 was estimated to be moderate (Oliva et al, 2006). Hence, one possible effect of a partial methylation of A58 in tRNA$^{Ser(UCN)}$ could be its involvement in stabilization of a tRNA pool that can be transient or tissue-specific. In support of that, the extent of m^{1}A58 modification significantly increased after treatment of cells with high doses of EtBr, possibly as an initial response to the blockage of transcription.

6.2.2 Effect on tRNA aminoacylation

The 7472insC mutation produced a 25% relative decrease in aminoacylation that may be rather important in combination with the low steady-state levels of the mutant tRNA$^{Ser(UCN)}$. Our assay did not compare directly the efficiency of aminoacylation. However, a construct of bovine tRNA$^{Ser(UCN)}$ with the mutation corresponding to the 7472insC was aminoacylated by recombinant bovine SerRS with the same efficiency as wild-type substrate (Shimada et al, 2001). From the relative estimation of aminoacylation it is also not possible to judge whether the mutant tRNA$^{Ser(UCN)}$ can act as a competitive inhibitor of wild-type tRNA, as was shown to occur in the case of mutations in tRNA^{Ile} with reduced catalytic efficiencies (Kelley et al, 2001).

Surprisingly, a comparable decrease in aminoacylation was reported for the A7445G mutation (Li X et al, 2005). Probably, other factors than the tRNA structure may contribute to such a decrease. Although the 7472insC mutation produces aberrant processing products at high frequency (III) which may be not charged efficiently, in the case of the A7445G mutation possible 3′-C miscleavage should be corrected by the formation of a functional CCA end (Yan H et al, 2006). Alternatively, some tRNA modifications essential for aminoacylation may be dependent on efficient tRNA processing, although this is unlikely to be of major importance, since the unmodified transcript can be charged *in vitro*.

Both mutations may significantly affect the kinetic equilibrium of aminoacylation *in vivo* due to reduced levels of tRNA$^{Ser(UCN)}$. While this does not seem to be a common reason for inefficient charging of mutant tRNAs, a low number of cognate tRNA molecules might impair the accuracy of aminoacylation by SerRS. Due to a similarity it the T-loop sequence, tRNA^{Gln} can also be recognized by SerRS and significantly misacylated *in vitro* (Shimada et al, 2001). The kinetic discrimination of non-cognate tRNA is suggested to maintain the fidelity of mitochondrial translation, possibly also in the case of other mitochondrial aminoacyl-tRNA synthetases. However, no examples of misacylation have been found so far *in vivo*. The accuracy of translation may be further controlled by less efficient accommodation of misacylated tRNA at the ribosome due to altered interaction with EF-Tu (LaRiviere et al, 2001; Asahara and Uhlenbeck, 2002).

Aminoacylation of human mitochondrial tRNA$^{Leu(CUN)}$ is proposed to be self-regulating, due to a unique ability to switch between two conformations resulting from a slip in the flexible T-stem (Hao et al, 2005). Such a mechanism possibly has a special role in the regulation of aminoacylation of leucine-isoaccepting tRNAs. Interestingly, an extra G inserted by the 7472 mutation would allow a similar T-stem slip in tRNA$^{Ser(UCN)}$. However, most probably this is thermodynamically unfavourable, due to a high GC content and stable structure of this region confirmed by our analysis. Nevertheless, the mutation is likely to *reduce* the conformational flexibility of tRNA$^{Ser(UCN)}$ if it creates an extra pair in the T-stem. Although this does not have a dramatic effect on aminoacylation, in other processes, such as interaction with maturation enzymes and functioning at the ribosome, optimal flexibility of tRNA might be crucial. The molecular mechanism of action of mutations that improve aminoacylation through stabilization of the T-stem, such as the C4320T mutation in tRNA^{Ile} (Kelley et al, 2001) and the T12311C mutation in tRNA$^{Leu(CUN)}$ (Hao et al, 2005), could be associated with increased structural rigidity.

Aminoacylation itself induces conformational changes in tRNAs (Enriquez and Attardi, 1996; Sohm et al, 2004). For tRNA$^{Ser(UCN)}$, this change might be rather minor, since in combination with a small amino acid it is not enough to retard significantly the migration of charged tRNA in acidic gels (I). For other tRNAs, such as tRNA$^{Leu(UUR)}$ and tRNA^{Lys}, proper folding of which depends on certain modifications (Sohm et al, 2003; Helm et al, 2004), the conformation upon aminoacylation is changed significantly. A high structural flexibility might partially explain why these tRNAs are particularly sensitive to the effects of pathogenic mutations.

Aminoacylation of serine-isoaccepting tRNAs

In human mitochondria, the two serine-isoaccepting tRNAs are present at nearly the same abundance, whereas AGY codons are less frequently used in translation than UCN codons (King and Attardi, 1993). Although there is no

information about the relative aminoacylation levels of the isoacceptors *in vivo*, this balance might depend on several factors, the rate of deacylation in translation being probably the main one. The discriminator base (N73) is a strong recognition element in most tRNAs. The nature of discriminators in serine-isoaccepting tRNAs in human and bovine mitochondria is different: whether G or A. Since bovine SerRS has a preference for G73, which is the discriminator base of tRNA$^{Ser(AGY)}$ with its unusual structure, this may adjust the balance of aminoacylation of the two isoacceptors (Shimada et al, 2001). Interestingly, the discriminators of human serine-isoacceptors are reciprocally opposite, so that tRNA$^{Ser(UCN)}$ has the more efficient discriminator G73. Although the preference of human SerRS might be different, the nature of the discriminator in case of tRNA$^{Ser(UCN)}$ seems to correlate with the occurrence of the m^{1}A58 modification, which is normally present in the bovine (Yokogawa et al, 1991) but not the human tRNA (see previous section). The A58-U54 pair is proposed to be a strong recognition element for serylation of both isoacceptor tRNAs by bovine SerRS (Ueda et al, 1992; Shimada et al, 2001). The interaction within this pair, however, may be of special importance for aminoacylation of tRNA$^{Ser(UCN)}$, since A58 in tRNA$^{Ser(AGY)}$ is recognized rather base-specifically (Shimada et al, 2001). Therefore, the strength of the A58-U/T58 interaction, possibly modulated by the m^{1}A58 modification, is likely to influence the efficiency of aminoacylation through stabilization of tRNA structure. The occurrence of this modification at a low frequency in human tRNA$^{Ser(UCN)}$ might be involved in regulation of the balance of aminoacylation.

A possible excess of charged tRNA$^{Ser(AGY)}$ compared to its infrequent usage in translation might be required to compensate for its low activity in the formation of ternary complex with EF-Tu observed *in vitro* (Hunter and Spremulli, 2004). Interestingly, the proportion of tRNA^{Phe} able to form the EF-Tu complex was even lower, and the ternary complex was relatively weak. Since this is one of the most abundant tRNAs in human mitochondria (King and Attardi, 1993), it might be speculated that ternary complex formation may balance the amount of tRNAs active in translation that would be otherwise present in excess, taking into account a limited concentration of EF-Tu (Cai et al, 2000). To some extent, interaction with EF-Tu might regulate tRNA turnover by protecting it from degradation. Moreover, human mitochondrial PheRS has a significantly lower affinity for tRNA compared to that of the prokaryotic and cytosolic enzymes (Bullard et al, 1999). Since the amount of aaRSs could also be limiting, aminoacylation may be one of the stabilizing factors involved in the adjustment of tRNA steady-state levels (King and Attardi, 1993). In that sense, regulation of aminoacylation efficiency by the m^{1}A58 modification would have also an indirect effect on tRNA$^{Ser(UCN)}$ stability.

6.2.3 Effect of EF-Tu overexpression

In my analysis, overexpression of EF-Tu did not improve the steady-state levels of the mutant tRNA$^{Ser(UCN)}$, in agreement with the observation that the mutation results in no increase in tRNA turnover. Nevertheless, it could have had a beneficial effect on mitochondrial protein synthesis, which was not tested. Although overexpression of EF-Tu can complement mitochondrial tRNA mutations in yeast (Feuermann et al, 2003), it may not be regarded as a universal 'strategy'. First, there can be species-specific differences in abundance of EF-Tu and kinetics of ternary complex formation (Rosenthal and Bodley, 1987; Cai et al, 2000). In addition, *S. cerevisiae* uniquely lacks the mitochondrial exchange factor EF-Ts, which can probably be compensated by the ability of EF-Tu to self-exchange, due to its low affinity for GDP (Rosenthal and Bodley, 1987; Chiron et al, 2005).

Second, although the structure of EF-Tu is highly conserved, human mitochondrial tRNAs deviate from classical ones (Helm et al, 2000), which might imply some difference also in their interaction with EF-Tu. For example, a rather small fraction of mitochondrial tRNAs appears to maintain a stable structure that can be recognized by EF-Tu (Hunter and Spremulli, 2004).

Third, the interaction of EF-Tu with aminoacylated tRNA is proposed to be thermodynamically compensated by different contributions of the tRNA body and the amino acid to the overall binding affinity (LaRivier et al, 2001). Mutations in tRNAs with a weak contribution might be better compensated by more stable interaction of EF-Tu with the cognate amino acid.

Finally, there are other factors that determine the half-life of tRNA, one of which could be the rate of deacylation, i.e. the lability of the aminoacyl ester bond. In my experiments, when mitochondrial transcription was blocked, tRNA^{Lys} was partially deacylated over time, whereas aminoacylated tRNA$^{Leu(UUR)}$ was much more stable.

Inefficient ternary complex formation could be a potential pathological mechanism of tRNA mutations by affecting the steady-state levels of tRNA and the accuracy of translation. In two studies performed so far, mutant tRNAs were proposed to have a decreased affinity for EF-Tu, resulting in less efficient protection against degradation (Yasukawa et al, 2001; Hino et al, 2004). In general, since most of the mitochondrial EF-Tu is estimated to be present in a bound state (Cai et al, 2001), its overexpression might still improve the efficiency of ternary complex formation in some cases.

6.3 Molecular effects of the 7472insC mutation on tRNA biogenesis (II, III)

The low steady-state levels of the mutant tRNA$^{Ser(UCN)}$ could be caused, in theory, either by increased turnover or by a decreased rate of synthesis. Based on my measurements, the half-life of the mutant tRNA was not affected. However, the rate of its synthesis was reduced sufficiently to provide a potential explanation for the comparative deficiency of the mutant tRNA. The levels of other tRNAs from the L-strand transcriptional unit were unaffected, indicating that the effect of the mutation on tRNA synthesis was posttranscriptional. We thus considered a posttranscriptional defect in tRNA synthesis as a plausible candidate for the major mechanism of the mutation. Subsequent studies (III) revealed more specific effects. The mutation was found to interfere with both 5´- and 3´-processing, causing a high frequency of misprocessing *in vivo* and a decreased efficiency of processing *in vitro*, influenced by the order of cleavage.

6.3.1 Interference with RNA processing

The analysis of processing products *in vivo* revealed that the most frequent and the most specific defect found uniquely in mutant cells was a miscleavage at 5´ U(-1), either alone or in combination with 3´-misprocessing. Such molecules are likely to arise from miscleavage by RNase P. This was not, however, observed *in vitro* using a substrate already preprocessed at the 3´-end. Importantly, the natural transcript, which is several kilobases long, may have a much more complex structure. The 7472insC mutation could also interfere specifically with certain base modifications or stabilizing factors that might be required for accurate processing *in vivo*. Miscleavage by RNase P might be either primary or, alternatively, facilitated by inaccurate 3´-processing. In the latter case, it is likely that additional nucleotide(s) retained at the 3´-end could favour the interaction between the discriminator G73 and U(-1), masking the correct recognition site for RNase P (Fig. 6.1a). In support of that, the mutant construct with incorrect UCCA 3´-end was processed less efficiently in my analysis than wild-type, and sometimes 5´-misprocessed in the predicted fashion.

Similarly, the discriminator C73 forming a base pair with G(-1) in bacterial tRNA^{His} results in an unusual cleavage at N(-1), generating a 8 bp acceptor stem (Burkard et al, 1988). Such base pairing is proposed to determine the cleavage site as a result of C73 interaction with U294 in M1 RNA (Brännvall et al, 2002). It should be noted, however, that interaction with the RCCA element (Fig. 6.1b) is supposedly not required for human RNase P, due to the absence of the L15 region (Frank et al, 2000). Although in bacteria the interaction between N(-1) and A248 is universal and may explain a conserved preference for U(-1) (Zahler et al, 2003; Brännvall et al, 2002) (Fig. 6.1b), A248 is not present in eukaryotic RNase P (Frank et al, 2000). If, nevertheless, U(-1) is preferable, the interaction

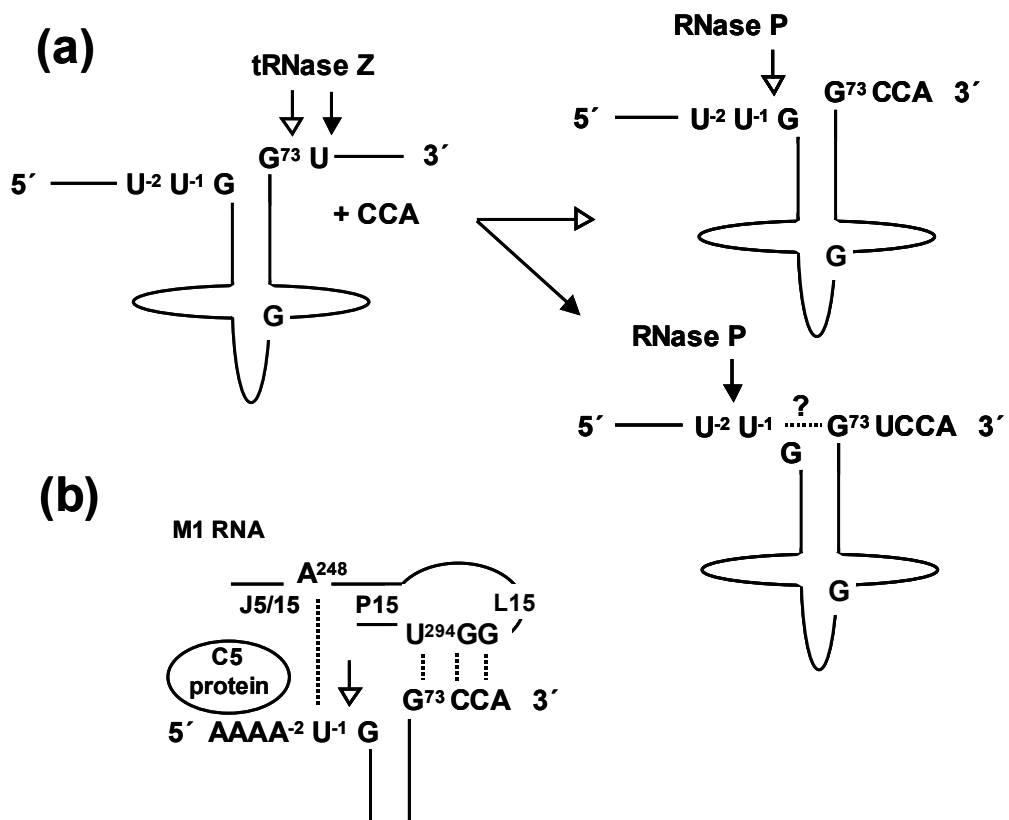with U(-2) in the mutant tRNA might be equivalent to the normal U(-1) interaction and result in miscleavage.

Figure 6.1. *Possible interference of the 7472insC mutation with processing of pre-tRNA$^{Ser(UCN)}$ and recognition of cleavage site by bacterial RNase P. (a) 3´-misprocessed products could be created by tRNase Z miscleavage or by alternative exonucleases (not shown). Miscleavage at the 3´-end might favour the G73 – U(-1) base pair formation, facilitating 5´-misprocessing. The sites of correct and incorrect processing are indicated by open and filled arrowheads, respectively. (b) Interactions between bacterial RNase P M1 RNA and pre-tRNA are indicated by dashed lines (modified from Harris and Christian, 2003 and Zahler et al, 2003). The cleavage site is indicated by an arrow.*

RNase P interaction with tRNA and subsequent cleavage is based on multi-step conformational changes (Zahler et al, 2005). According to a threshold model for loss of cleavage fidelity, destabilization of the substrate-enzyme complex over a certain threshold, in combination with the disruption of the N(-1)/A248 interaction, would result in miscleavage (Zahler et al, 2005). Applying this knowledge to tRNA point mutations, any changes in the kinetics of the 'correct' RNase P reaction might potentially produce a miscleavage. At least in tRNA$^{Ser(UCN)}$ and tRNA$^{Leu(UUR)}$ such misprocessing might be favoured by possible interaction of U(-1) with the discriminator and the presence of U(-2).

Besides changes in the substrate, the efficiency and accuracy of processing *in vivo* may depend on multiple factors. The amount of processing enzymes is likely to be one of them, and the RNase P concentration in human cells is estimated to be very low (Puranam and Attardi, 2001). In the mitochondria of mutant cells, this amount could be even lower if the efficiency of protein import in general is decreased. Ionic composition could perhaps also influence processing efficiency. An example of an association of a tRNA point mutation with hypomagnesemia is reported by Wilson et al (2004), although this cannot be directly applied to the ionic environment inside mitochondria.

RNase P catalysis is strongly dependent on divalent metal ions, particularly Mg^{2+}, which is bound in the vicinity of the cleavage site (Kirsebom 2002; Brännvall and Kirsebom, 2005). Both bacterial and eurakyotic enzymes have been shown to be inhibited by aminoglycosides *in vitro*, which compete with Mg^{2+} for common binding sites (Mikkelsen et al, 1999; Tekos et al, 2000). This raises a hypothetical possibility that, besides inhibition of translation due to the binding to 12S rRNA (Hamasaki and Rando, 1997), aminoglycosides might interfere with tRNA biogenesis *in vivo*. In cybrid cells with the A1555G mutation, a growth defect in the presence of aminoglycosides was not associated with a detectable respiratory chain dysfunction or a decrease in mitochondrial protein synthesis rate (Giordano et al, 2002). However, the side-effects of aminoglycosides may be rather complex, since they inhibit several other catalytic RNAs, such as self-splicing group I introns and hammerhead ribozymes (Schroeder et al, 2000). The ototoxic effect of aminoglycosides is not always related to the A1555G mutation. In a set of 45 patients with aminoglycoside-induced deafness no A1555G mutation was found, yet 3 patients with the 7472 mutation were reported as having developed acute hearing loss after the use of streptomycin (Verhoeven et al, 1999). Aminoglycosides were found to act as modifiers also of the G7444A mutation that had otherwise a very low penetrance of hearing impairment (Zhu et al, 2006). Although the 3′-processing defect detected *in vitro* (Yan H et al, 2006) could be the primary effect of this mutation, the misprocessed protruding 3′-U might interfere also with 5′-processing by the mechanism suggested above.

6.3.2 Processing pathway of tRNA^{Ser(UCN)}

In my analysis, the efficiency of 3′-processing of the mutant substrate *in vitro* was strongly dependent on the order of processing, implying that the effect of the mutation *in vivo* should depend on the processing pathway. Most tRNAs are believed to be processed at the 5′-end first. Such priority could be enforced by the structure of the substrate, tRNase Z being inhibited by long 5′-extensions (Nashimoto 1999; de la Sierra-Gallay et al, 2005). In addition, several other factors might regulate the order of processing, for example, the relative concentration of processing enzymes. In yeast, tRNA^{Trp} is processed at the 3′-end first, proposed to be due to either an unidentified factor binding this tRNA or

to the intrinsic structure of the precursor (Kufel and Tollervey, 2003). Human La protein is suggested to control 5´-processing by blocking the site of RNase P cleavage in a phosphorylation-dependent manner (Intine et al, 2000). Such factors directing the order of processing could exist also in mitochondria, and their binding might be potentially altered by tRNA mutations. The processing of tRNA$^{\text{Ser(UCN)}}$ might be unusual due to long non-coding sequences in the precursor that may require stabilizing factors or provide intrinsic structure.

The order of processing of tRNA$^{\text{Ser(UCN)}}$ has been hypothesized to occur first at the 3´-end, since the A7445G mutation, which blocks 3´-processing *in vitro* (Levinger et al, 2001), leads to a decrease also in ND6 mRNA levels (Guan et al, 1998). The T7511C mutation, presumably interfering only with 5´-processing, does not affect ND6 levels (Li et al, 2004). Nor does the 7472insC mutation, although according to my *in vitro* experiments, the 3´-processing defect is nevertheless somewhat more significant quantitatively. However, this may not accurately reflect the situation *in vivo* or in all tissues. Also, the degree of the processing defect did not correlate with the decrease in ND6 levels in two different cell lines (Guan et al, 1998; Li X et al, 2005). The pattern of misprocessed products found in my work *in vivo* does not prove the order of processing of tRNA$^{\text{Ser(UCN)}}$, since the defects at the 5´-end may have less chance to be repaired than the defects at the 3´-end. However, it could be suggested that any priority of 3´-end processing may produce more severe effects on the levels of both tRNA$^{\text{Ser(UCN)}}$ and ND6 mRNA. The exact mechanism of ND6 mRNA processing is unknown, however, and might involve also alternate pathways and activities. It is likely that the ND6 mRNA abundance is dependent on the processing pathway also in case of other mutations in tRNA$^{\text{Ser(UCN)}}$. A different order of processing in muscle and fibroblasts has been observed in the case of the A3302G mutation, resulting in a tissue-specific accumulation of RNA intermediates (Bindoff et al, 1993). More generally, mitochondrial tRNA mutations interfering with processing may contribute to a tissue-specific phenotype by affecting also the levels of neighbouring mRNAs and/or rRNAs, depending on the exact order of processing.

6.3.3 Interference with other maturation steps

The decreased efficiency of RNA processing could be the major but not the only mechanism leading to the low steady-state levels of the 7472insC mutant tRNA$^{\text{Ser(UCN)}}$. In fact, any step of tRNA biosynthesis could be sensitive to structural rearrangements. For example, CCA addition has been shown to be sensitive to mutations in the acceptor and T-arm (Tomari et al, 2003; Levinger et al, 2004b). In my study, the extent of incomplete CCA sequences in mutant tRNA$^{\text{Ser(UCN)}}$, however, was the same as in wild-type tRNA and there was no accumulation of shorter forms of mutant tRNA.

It is important to mention that our analysis of tRNA structure was performed on molecules presumably stable as a result of successful maturation. However,

an increased degradation of unmodified molecules, such as suggested to occur in the case of the T7512C and G7497A mutations (Möllers et al, 2005), cannot be excluded.

As mentioned in the previous section, the 7472insC mutation might produce minor rearrangements in the T-loop and possibly creates an extra base pair in the T-stem, concomitantly reducing the size of the T-loop. The conformation of the T-loop maintained by the G53-C61 base pair and its correct size of 7 nt are critical for the formation of both T54 and ψ55 modifications by yeast enzymes (Becker et al, 1997). Moreover, both modification enzymes *per se* may have a protective role in the maturation process independent of catalysis, acting as RNA chaperones (Hoang and Ferre-D'Amare, 2001; Johansson and Byström, 2002). ψ55 synthase is suggested to access U55 more easily in the so-called λ-form in which tertiary interactions are disrupted (Ishitani et al, 2003). The transition between the L- and λ-forms might be a rate-limiting step of the modification reaction (Ishitani et al, 2003). Thus, the efficiency of the reaction is likely to depend on the global tRNA conformational flexibility.

T54 and ψ55 belong to class I modifications, which occur at an early stage in tRNA maturation, in order to assure correct tRNA folding (Grosjean et al, 1996; Helm 2006). Therefore, failure of these core modifications may result in rapid tRNA degradation. Pseudouridylation is suggested to determine how long tRNA is held open by ψ55 synthase (Hoang and Ferre-D'Amare, 2001). This interaction might act as a quality control step ensuring either correct folding or exposing tRNA to degradation. Moreover, yeast tRNA ψ55 synthase (encoded by PUS4) is proposed to compete with RNase P for binding to certain tRNA precursors when overexpressed (Qiu et al, 2000). This raises a possibility that inefficient catalysis of the ψ55 isomerization might interfere also with 5´-processing, depending on which of these processes occurs first. In bacteria and yeast, aberrant pre-tRNAs are degraded through polyadenylation (Li Z et al, 2002; Kadaba et al, 2004). As I discuss below, a similar mechanism may exist also in human mitochondria.

A different mechanism of rapid degradation of pre-existing mature tRNAs lacking functionally non-essential modifications has recently been proposed by Alexandrov et al (2006). A fraction of unmodified tRNA, however, remained stable, probably due to binding to a protective component such as aaRS or due to its sequestration in a distinct subcellular compartment. Similarly, a persistent stable pool was observed for both mutant and wild-type tRNA[Asn] after degradation *in vitro* (Hao and Moraes, 1997). This might resemble a common situation of mutant mitochondrial tRNAs, with low steady-state levels probably represented by a stable tRNA subpopulation.

As could probably be generalized, interaction with enzymes and protein factors (such as EF-Tu) may have a stabilizing effect on tRNA at different steps of biogenesis. In yeast, La protein, which acts to protect pre-tRNAs from exonucleolytic degradation during 3´-processing (Yoo and Wolin, 1997), is required also for correct folding of certain pre-tRNAs (Chakshusmathi et al, 2003). La protein is suggested to be one of the factors contributing to tRNA

stability, acting redundantly with tRNA modification enzymes (Copela et al, 2006). Such tRNA chaperone-like proteins might be expected to exist also in mitochondria.

6.4 Polyadenylation of human mitochondrial tRNAs

The key steps in tRNA maturation that are highly dependent on correct tRNA structure might be considered also as tRNA quality checkpoints, resulting in rapid degradation of defective tRNAs. This could be one of the common reasons for decreased steady-state levels of mutant mitochondrial tRNAs in cases of diseases. Efficient aminoacylation may finally ensure functional tRNA selection. A turnover mechanism should exist also for aberrant tRNAs, which are otherwise stable. In my unpublished work, I provide the first evidence of such a mechanism in human mitochondria.

6.4.1 The tRNA polyadenylation phenomenon

As I show, in response to high doses of EtBr, mitochondrial tRNAs appeared to become polyadenylated. What is most probably a similar effect was first reported by Hirsch and Penman (1974), who observed post-transcriptional addition of poly(A) tails to short pieces of RNA in the presence of 1 μg/ml EtBr. Based on the estimated size of 4-6 S, this RNA fraction should correspond to polyadenylated tRNA products as found in my work, with lengths up to 200 nt.

Since EtBr interferes specifically with mitochondrial nucleic acids (e.g. Leibowitz, 1971 and references therein), it could presumably affect the normal tRNA structure, consequently preventing its aminoacylation. Obviously, only deacylated tRNA could be a substrate for polyadenylation. More specifically, the preferential intercalation site of EtBr into tRNA appears to be between base pairs 6 and 7 at the bottom of the acceptor stem (Wells and Cantor, 1977; Chu et al, 1997). This site could be determined by a unique combination of ribose 2´-endo conformation and double strandedness which, as a structural feature common to all tRNAs, is hypothesised to be recognized by many of the proteins involved in tRNA interactions (Wells and Cantor, 1977). One universal such factor might be EF-Tu, which binds to one side of the acceptor helix (Nissen et al, 1996). Moreover, base pair 7-66 could be one of the determinants affecting bacterial EF-Tu binding affinity (Asahara and Uhlenbeck, 2002). The A4269G mutation in tRNA[Ile] at position 7 has been demonstrated to decrease mitochondrial EF-Tu binding affinity (Hino et al, 2004).

The extent of polyadenylation, however, was different for eight studied mitochondrial tRNAs (see Table 5.4). The reason for this is not clear, but could depend on the combination of tRNA primary sequence, structure, abundance and localization, as well as the presence in complexes with aaRS and EF-Tu, which

might prevent EtBr intercalation. The availability of different tRNAs for polyadenylation might also be partly determined by the rate of deacylation during translation, which would depend, in turn, on the frequency of codon usage. A crude such correlation could be found for several tRNAs, including the seryl-tRNA isoacceptors, with the more infrequently used tRNA$^{Ser(AGY)}$ (King and Attardi, 1993) being almost resistant to polyadenylation. As another example, tRNA^{Phe} was particularly sensitive to the EtBr effect, possibly because of its relatively high abundance (King and Attardi, 1993), in combination with a weak ternary complex formation (Hunter and Spremulli, 2004), which might increase the relative amount of the tRNA in the unbound/ deacylated state.

In the absence of EtBr, polyadenylated products were rapidly degraded. Their half-life of about 60-90 min is comparable with that of mitochondrial mRNAs, which is between 25 and 90 min (Gelfand and Attardi, 1981). The role of polyadenylation in mitochondrial RNA turnover is variable in different organisms. In human mitochondria, mRNAs are polyadenylated (Ojala et al, 1981). Long poly(A) tails, however, may be not required for mitochondrial mRNA stability (Tomecki et al, 2004). On the other hand, some mitochondrial mRNAs could be stabilized by polyadenylation (Nagaike et al, 2005). In my work, products with long poly(A) tails appeared to be more stable than oligoadenylated tRNAs, based on their different rate of turnover. Interestingly, the length of poly(A) tails of the most stable products was different for individual tRNAs. It should be noted, however, that the poly(A) tails may be extended to the maximal length only in the presence of EtBr, due to the apparent inhibition of degradation, which may normally compete with their synthesis.

The stabilizing effect of polyadenylation could be provided by poly(A)-binding proteins, so far not identified in mitochondria. An optimal length of the tails may be required for interaction with any such factors. According to a model for turnover of *T. brucei* mitochondrial *rps12* RNA, a poly(A)-binding protein, which requires only a short tail, recognizes also a small edited element present at the 3´-end, determining RNA stability or degradation (Kao and Read, 2005). Thus, other factors than the presence of poly(A) tails alone might be involved in regulation of specific RNA turnover.

The turnover of polyadenylated tRNA products was accompanied by gradual recovery of tRNA levels. This could be a result of degradation of poly(A) tails or, alternatively, tRNA synthesis *de novo*. The high concentration (1 μg/ml) of EtBr did not cause mtDNA degradation in HeLa cells, at least during the first hours of treatment (Leibowitz, 1971). Persisting mtDNA could therefore possibly function as a template for transcription after removal of the drug.

In yeast, the complete degradation of tRNA is suggested to require a cooperation of polyadenylation and degradation complexes and may involve multiple rounds (Vanacova et al, 2005). Accordingly, multiple sites of internal polyadenylation have been reported for human mitochondrial RNA, including tRNA^{Lys} (Slomovic et al, 2005). My results imply that the initiation of tRNA polyadenylation might be rather inefficient, starting with a time lag after deacylation. After 4 hours of incubation with EtBr, when partial polyadenylation

was observed, tRNA was completely deacylated (Fig. 5.17a). Polyadenylation of tRNA substrate by bacterial PAP I *in vitro* appeared to be biphasic, with a slow rate of addition of the first few A residues (Feng and Cohen, 2000). Thus, it is possible that polyadenylation competes with other processes at the 3′-end of tRNA, such as CCA addition and aminoacylation, which are normally much more efficient. Polyadenylation might serve then as a backup system, providing tRNA a possibility to be aminoacylated once the tail is removed.

6.4.2 tRNA polyadenylation and turnover enzymes

The enzymes involved in tRNA polyadenylation and degradation remain to be identified. The obvious candidates would be PAP and PNPase, both identified in human mitochondria (Piwowarski et al, 2003; Tomecki et al, 2004) and proposed to regulate mitochondrial mRNA stability (Nagaike et al, 2005). The rather homogeneous composition of poly(A) tails in human mitochondria strongly suggests that they are synthesized by PAP (Tomecki et al, 2004; Slomovic et al, 2005). In my work, the frequency of incorporation of non-A residues was 4-5%, in contrast to only 0.6% reported for mitochondrial mRNAs (Tomecki et al, 2004). This frequency, however, is much lower than for chloroplast PNPase, which creates heterogeneous purine-rich tails containing 70% adenosines and 25% guanosines (Lisitsky et al, 1996). Interestingly, both homogeneous and heterogeneous poly(A) tails are present on nuclear-encoded rRNA fragments in human cells, implying that either PAP or PNPase might be involved in their synthesis, possibly acting in different compartments (Slomovic et al, 2006). The identity of mitochondrial tRNA polyadenylation enzymes could be addressed further by RNA interference experiments.

In yeast, a defective pre-tRNA, as well as several noncoding RNAs, are polyadenylated by the nuclear TRAMP complex containing a novel PAP, Trf4p (Vanacova et al, 2005; LaCava et al, 2005; Wyers et al, 2005). The important difference between Trf4p and the classical PAP is a low processivity of Trf4p, which creates oligo(A) tails probably too short to bind the stabilizing poly(A)-binding protein Pab1b (LaCava et al, 2005). Moreover, a distinct polymerase, Trf5p, may function in a related complex with different substrate specificity (Houseley and Tollervey, 2006). Such examples leave a hypothetical possibility that distinct enzymes could be involved also in polyadenylation of coding and non-coding mitochondrial RNAs.

Interestingly, in maize mitochondria, CCA-like sequences were found at the 3′-end of truncated mitochondrial *rps12* mRNA, possibly created by a tRNA nucleotidyltransferase-related enzyme (Williams et al, 2000). Similar extra nucleotides were present at the 3′-end of *Arabidopsis* mRNAs, upstream of poly(A) sequences (Jin and Bian, 2004). This suggests that CCA- and poly(A)-adding enzymes may cooperate or, alternatively, compete during the 3′-end processing of plant mRNAs. Free CCA ends of tRNAs might thus serve as primers for poly(A) addition.

Human mitochondrial PNPase is proposed to function as an exonuclease (Nagaike et al, 2005) and therefore could mediate mRNA degradation, similarly to a component of the yeast degradosome Dss1p (Gagliardi et al, 2004). The involvement of PNPase in polyadenylation-dependent degradation of tRNA in bacteria (Li Z et al, 2002) and several non-coding transcripts in *Arabidopsis* mitochondria (Holec et al, 2006) may imply its similar role in non-functional tRNA turnover.

6.4.3 Towards the mechanism of tRNA polyadenylation

The analysis of human mitochondrial transcripts revealed that, in addition to stable polyadenylation, all types of RNA are internally polyadenylated at low abundance (Slomovic et al, 2005). Similar non-abundant polyadenylated fragments were found for human nuclear-encoded 18S and 28S rRNAs (Slomovic et al, 2006). Such truncated molecules are likely to be substrates for degradation. My results suggest that a polyadenylation-dependent mechanism of degradation is involved also in a quality control of aberrant tRNAs in human mitochondria, which are otherwise stable.

The common feature of stable RNA substrates for polyadenylation-dependent degradation could be their non-functionality due to structural abnormality or lack of protective factors. As a result of inefficient competition with the normal maturation process, the 3′-ends of such molecules could become exposed to polyadenylation as the first step in this pathway.

For mature tRNAs, efficient aminoacylation is likely to be the main competitor with polyadenylation. According to the model of a channelled tRNA cycle, the mammalian translation system is organized to transfer tRNA directly to its cognate aaRS without dissociation into the surrounding medium (Stapulionis and Deutscher, 1995). eEF1A could play a special role in such channelling, since it is able to form a non-canonical tertiary complex with deacylated tRNA released from the E site of the ribosome, and a subsequent quaternary complex with aaRS (Petrushenko et al, 2002). Applying this model to the mitochondrial translation system, stable complex formation with EF-Tu during the ribosome cycle might be considered as a critical step in tRNA quality control. As I mention in my summary of the results (5.4.3), my studies strongly imply that tRNA polyadenylation is associated with the ribosome. Moreover, there is a hypothetical possibility for direct involvement of EF-Tu in tRNA degradation, if aminoacylation fails. For example, eEF1A can recognize damaged proteins that are then ubiquinated, facilitating their delivery to the proteasome (Chuang et al, 2005).

In theory, the mechanism of polyadenylation-dependent degradation may also apply to mitochondrial tRNA mutations not affecting tRNA stability, but which cause tRNA dysfunction mainly in translation. Mutations with mild molecular effects, like the 7472insC mutation, would be plausible candidates to cause a decrease in tRNA steady-state levels partially through this pathway. On

the other hand, some pathological tRNA mutations might impede polyadenylation-dependent degradation, the extent of which appeared to be tRNA substrate-dependent at least in studies using EtBr. Moreover, a similar mechanism (but not linked to translation) is likely to eliminate poorly processed mutant tRNA precursors.

Summary

Thus, the main molecular effect of the 7472insC mutation is a dramatic decrease in the steady-state levels of tRNA$^{Ser(UCN)}$. The major mechanism causing this decrease is inefficient tRNA synthesis due to interference with several steps of RNA processing. This is the first revealed mitochondrial tRNA mutation that affects both 5′- and 3′-processing efficiency and accuracy. Moreover, the effects on 3′-cleavage are strongly dependent on the order of processing. Since the processing pathway may vary between different tissues, it could result in tissue-specific effects of the mutation *in vivo*.

Although the effects of the 7472insC mutation on tRNA structure and function appeared to be minimal, a 25% defect in aminoacylation could be rather significant in combination with the low steady-state levels of the mutant tRNA$^{Ser(UCN)}$. The resulting mechanism of the 7472insC mutation could be, therefore, considered as cumulative. In addition, a threshold for expression of the effects of the mutation could depend on tissue-specific factors plus genetic and environmental modifiers.

Aberrant mitochondrial tRNAs are degraded through polyadenylation. It could be a common mechanism of tRNA quality control in mitochondria, possibly operating also in the case of tRNA point mutations.

7. CONCLUSIONS AND PERSPECTIVES

Mitochondrial tRNA$^{Ser(UCN)}$ is a hot spot for deafness-associated mutations. My research concerns one of them, the 7472insC mutation. In my studies, I have characterized the molecular phenotype of this mutation in cybrid cell lines and analysed its effect on tRNA structure and biosynthesis. The findings lead me to propose a molecular pathogenic mechanism for the 7472insC mutation. In addition, I found some evidence for a specific mechanism of elimination of defective mitochondrial tRNAs. My main conclusions are the following:

1) The main effect of the 7472insC mutation in osteosarcoma cell cybrids is a ~65% decrease in the steady-state level of tRNA$^{Ser(UCN)}$. This decrease is associated only with a small quantitative defect in mitochondrial protein synthesis. In the presence of doxycycline, however, this effect is more pronounced. The 7472insC mutation has a mild effect on the ability of cells to grow under respiratory stress. The growth impairment could be significant in combination with a reduced mtDNA copy number. The clinical phenotype of the 7472insC mutation might thus depend on several modifying factors.

2) Human mitochondrial tRNA$^{Ser(UCN)}$ is base-modified at five positions. In addition, the adenine at position 58 could be methylated at low frequency under normal conditions. The 7472insC mutation does not change the steady-state pattern of base modifications. Nevertheless, interference with the efficiency of modification cannot be excluded. The mutation does not cause any major structural rearrangements in tRNA$^{Ser(UCN)}$, although the exact location of the additional nucleotide in the tertiary structure remains unresolved.

3) The 7472insC mutation produces a 25% relative decrease in aminoacylation *in vivo*. This defect could be significant in combination with the low steady-state level of mutant tRNA$^{Ser(UCN)}$.

4) The half-life of mutant tRNA$^{Ser(UCN)}$ is not decreased. Overexpression of mitochondrial EF-Tu has no effect on the steady-state level of the mutant tRNA in cultured cells.

5) The 7472insC mutation causes a significant posttranscriptional defect in synthesis of tRNA$^{Ser(UCN)}$ without a polarity effect on downstream-encoded tRNAs. The synthesis defect is attributed to an impairment in 5´- and 3´-processing. *In vivo,* mutant tRNA$^{Ser(UCN)}$ molecules are

misprocessed at high frequency (>11%) either at 5´-, 3´-, or both termini. *In vitro*, the mutation affects the efficiency of both 5´- and 3´-processing. The efficiency of 3´-cleavage is highly dependent on the order of processing. Misprocessing at the 3´-end could further interfere with the efficiency and accuracy of 5´-cleavage.

6) Therefore, the major mechanism of the 7472insC mutation is a multistep defect in RNA processing with the outcome depending on the processing pathway *in vivo*. The suggested mechanism could provide some explanations for the tissue-specific effects of the mutation. A similar mechanism could be applied to other mitochondrial tRNA point mutations.

7) Mitochondrial tRNAs with aberrant structure, which are otherwise stable, are eliminated by a polyadenylation-dependent degradation pathway. This tRNA quality control mechanism could possibly recognize also tRNAs bearing pathological point mutations.

My work aimed to characterize a detailed mechanism of the 7472insC mutation in a cybrid cell model. The decreased steady-state levels of the affected tRNA, which appeared to be the major molecular effect of the 7472insC mutation, is a typical sign of many other tRNA pathological point mutations (e.g. A7445G in tRNA$^{Ser(UCN)}$, A3243G in tRNA$^{Leu(UUR)}$, A4269G in tRNA^{Ile}). This common consequence could be a result of different molecular mechanisms, such as decreased tRNA stability or a defect in tRNA maturation. 7472insC is the first example of a mutation demonstrated to affect several interdependent RNA processing steps. With a further decrease in aminoacylation, a deficiency in the amount of functional tRNA$^{Ser(UCN)}$ may account for a tissue-specific defect in translation, which could be restricted mainly to cochlear cells, possibly due to a specific dependence on the UCN codons or some components of mitochondrial translation present in limiting amounts (Jacobs, 2003). A deficiency of tRNA$^{Ser(UCN)}$ could be regarded as the major molecular effect of several other mutations in this tRNA gene which associates them with sensorineural deafness, although the exact connection to the clinical phenotype so far is missing.

Understanding of the exact molecular mechanisms of tRNA pathological point mutations is essential for elaboration of efficient strategies for therapy. So far, this field remains poorly developed. Firstly, relatively few tRNA mutations have been studied in detail, and only some aspects of their molecular pathological mechanisms have been revealed. Second, there is a lack of general knowledge of human mitochondrial tRNA biology. For example, even the primary structure has been determined, including this study, only for 7 mitochondrial tRNAs (tRNA$^{Ser(AGY)}$, tRNA^{Pro}, tRNA^{Lys}, tRNA$^{Leu(UUR)}$, tRNA^{Ile}, tRNA$^{Ser(UCN)}$ and tRNA$^{Leu(CUN)}$) (Florentz et al, 2003; II; Kirino et al, 2006). As yet, very little is known about the biosynthesis of mitochondrial tRNA modifications. Further information will contribute to our understanding of the effects of pathogenic mutations on tRNA base modifications, some of which could be sensitive to (or affect) the global tRNA structure. Only a few human

mitochondrial aaRSs have thus far been cloned and characterized, and aminoacylation rules for mitochondrial tRNAs remain poorly understood. There are many open questions about tRNA processing activities, such as their identity, import into mitochondria, relative concentrations, tissue specificity, RNA substrate structural requirements and the possible existence of alternative processing pathways. The proposed mechanism of the 7472insC mutation may emphasize the need to address further studies on processing pathways of different mitochondrial tRNAs in different tissues. This general information, in combination with the available knowledge about the molecular pathological mechanisms of tRNA mutations, could help to predict the molecular effects of other mutations. Development of such criteria could be a next step after prediction of pathogenicity of the mutations, which has been addressed by several studies (Florentz and Sissler, 2001; McFarland et al, 2004; Kondrashov 2005).

The complexity of the relationship between molecular and clinical phenotypes requires further understanding of the involvement of genetic and environmental factors. In this direction, understanding of regulation of mitochondrial translation, screening for mutations in key components of tRNA metabolism, studies on their interference by clinically used drugs may provide some linkage to the variability of disease expression.

The components of the mitochondrial translation system might be considered also as targets for complementation strategies. As a first attempt, I have tested the effect of EF-Tu overexpression on the 7472insC mutation phenotype. Although it had no effect on the steady-state levels of tRNA$^{Ser(UCN)}$, there could be improvement in translation, especially under conditions when its efficiency is decreased, and this technique may be more successful particularly for structurally unstable mutant tRNAs. In general, this approach could be extended to the factors involved in tRNA biogenesis and translation (such as tRNA modification enzymes, aaRSs and other chaperones if they exist), whose concentration may be limiting in specific tissues. Understanding of the regulation of the import of RNA processing enzymes into mitochondria might provide some further possibilities for manipulation of their abundance.

The influence of various genetic factors on tRNA mutation phenotypes could be addressed by the creation of cybrid cell lines with different combinations of nuclear background and mitochondrial origin. In the future, transgenic mice may provide relevant disease models for studies on the mutation effects at the level of the whole organism, provided the modifiers hypothesised above operate similarly as in humans.

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ORIGINAL PUBLICATIONS

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The 7472insC mtDNA mutation impairs 5′ and 3′ processing of tRNA$^{Ser(UCN)}$

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Abstract

The deafness-associated 7472insC mtDNA mutation was previously shown to decrease the steady-state level of tRNA$^{Ser(UCN)}$ post-transcriptionally. To identify the affected tRNA maturation step(s) we analysed the effects of the mutation on processing in vivo and in vitro. tRNA$^{Ser(UCN)}$ from cybrid cells homoplasmic for 7472insC contained a high frequency (>11%) of molecules misprocessed at one or both termini. In vitro assays using partially purified HeLa cell RNase P and mitochondrial tRNA 3′ processing endonuclease (tRNase Z) confirmed that the efficiency of both 5′ and 3′ processing was impaired. A mutant precursor not already processed at the 5′ end was poorly processed in vitro by tRNase Z. Misprocessing at the 3′ end further impaired the efficiency and accuracy of 5′ processing of the mutant substrate. The mutation thus appears to affect several distinct, but interdependent, RNA processing steps, with the predicted outcome dependent on the exact processing pathway operating in vivo.
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Mitochondrial DNA mutations are associated with an increasing spectrum of human disorders, including both syndromic and non-syndromic forms of sensorineural deafness. One frequent class of such mutations affects mitochondrial tRNAs (see [1] for review). Various different molecular mechanisms appear to contribute to pathogenesis, including decreased aminoacylation, increased turnover, loss of base-modification, and defective 3′ end maturation. The common thread seems to be loss of function, typically associated with much reduced levels of the affected tRNA.

Several different mutations affecting the gene for tRNA$^{Ser(UCN)}$ have been found in multiple families showing deafness as a primary pathological feature [2–10]. The mutation A7445G, mapping one nucleotide beyond the 3′ end of the tRNA [2,3], is associated with the syndrome of sensorineural deafness and palmoplantar keratoderma [11]. The mutation 7472insC, which templates the insertion of an extra G into a run of 6 G residues located within the T-stem and extra arm of the tRNA [4,5], is associated with a syndromic disorder that includes hearing loss, ataxia, and myoclonus, but which manifests only as hearing impairment in most individuals. Other mutations in tRNA$^{Ser(UCN)}$, including T7512C, T7511C, and T7510C, have been reported in

families with syndromic or non-syndromic deafness [6–10].

In earlier studies we showed that 143B osteosarcoma cell cybrids with 100% 7472insC mutant mtDNA exhibited a mild deficiency of mitochondrial protein synthesis, associated with a 60–70% decrease in the steady-state level of tRNA$^{Ser(UCN)}$ [12]. This decreased tRNA abundance is attributable to a reduced rate of synthesis rather than increased turnover [13]. Since other tRNAs encoded within the light-strand transcription unit of mtDNA were essentially unaffected, we inferred that the 7472insC mutation must act post-transcriptionally [13].

Mitochondrial tRNA biosynthesis involves processing of a polycistronic transcript of each strand of the genome [14] by RNase P (5′ processing) and tRNA 3′ processing endonuclease (tRNase Z), both of which are encoded by nuclear genes that, at least in some species, give rise to the corresponding nuclear pre-tRNA processing enzymes [15,16]. In humans, a single type of RNase P, containing the catalytic H1 RNA, has been shown to be present both in the nucleus and the mitochondria [15]. The situation for tRNase Z is less clear, since the human genome has two genes encoding proteins of the tRNase Z family [16]. Other endonucleases and/or exonucleases have been postulated to play a role in mitochondrial tRNA processing, although they have not been characterized. The pathological A7445G mutation creates a tRNA$^{Ser(UCN)}$ precursor that cannot be processed by tRNase Z in vitro [17], yet does not result in a complete absence of the mature tRNA in vivo [18,19]. This indicates that an alternate 3′ end maturation pathway must exist, possibly akin to the exonucleolytic trimming seen in bacteria, in which at least five different exoribonucleases are involved [20].

The order of mitochondrial pre-tRNA processing is unclear and may vary between tRNAs, strands, cell-types, and/or species. In general, 5′ processing has been suggested to precede 3′ processing [21], but clear examples of the opposite pathway have been documented in mitochondria in humans in vivo and may vary between cell-types [22]. Following 3′ end processing, CCA is added by the mitochondrial isoform of the tRNA-3′ nucleotidyl transferase [23]. Finally, many base-modifications are effected. The stages at which they are carried out have not been clarified, and only a few of the enzymes involved are known. In the case of 7472insC, the pattern of base modification of tRNA$^{Ser(UCN)}$ in cybrid cells appears to be unaffected by the mutation [13]. However, any of the other steps in tRNA maturation might be impaired.

In order to elucidate more precisely the molecular mechanism of pathogenesis associated with 7472insC, we have analysed the products of pre-tRNA$^{Ser(UCN)}$ processing, both in vivo, in cybrid cells, and in vitro, using partially purified RNase P and tRNase Z.

Materials and methods

Cell-lines and cell culture. 143B osteosarcoma cell cybrids homoplasmic for the 7472insC mutation or for wild-type mtDNA from the same individual were as described previously [4,12]. Line 43 was used as the source of control tRNA and line 47 was used as the source of mutant tRNA. Cells were routinely cultured in DMEM supplemented with uridine and pyruvate as previously [12] and passaged weekly.

Oligonucleotides. Custom-designed oligonucleotides were purchased from DNA Technology (Aarhus, Denmark). Sequences are given in [13] except where stated (all shown as 5′–3′). For dot-hybridization, Ser11; for RT-PCR of circularized tRNA$^{Ser(UCN)}$, cser1, and cser2; for RT-PCR of non-circularized tRNA$^{Ser(UCN)}$, cser5—CAAAAAAGGAAGGAATCGAACC (np 7446–7467 of human mtDNA [24]) and cser6—GAAAAAGTCATGGAGGCCATG (np 7514–7494); and for fluorescent primer extension (minisequencing), cser3 and cser4. Oligonucleotides used for preparation of constructs for in vitro transcription are described in the relevant section, below.

Analysis of tRNA processing in vivo. Total RNA was extracted, using the Trizol method, from cells cultured in regular medium and from cells incubated in medium containing 250 ng/ml EtBr for two days and then allowed to recover in fresh medium for two days, as previously [13,12]. To deacylate tRNAs, 6 μg of total RNA was incubated in circularization buffer [25] for 10 min at 75 °C followed by 30 min at 37 °C. Deacylated tRNAs were circularized as described by Börner et al. [25], reverse-transcribed with primer cser1, and then amplified with primers cser1 and cser2 as previously [13]. Purified products were cloned using TOPO TA cloning kit (Invitrogen) and insert-containing colonies were identified by dot hybridization with oligonucleotide Ser11. Inserts were sequenced using standard dye-terminator technology, with M13 forward primer and analysis on an Applied Biosystems 3100 Genetic Analyzer using manufacturer's software.

Pre- tRNA$^{Ser(UCN)}$ constructs. Constructs were designed initially to yield exactly the same in vitro transcription products as previously tested as RNase P substrates by Puranam and Attardi [15], except for the insertion of the additional G templated by the 7472insC mutation in the mutant version. First, the tRNA$^{Ser(UCN)}$ region of mtDNA from both the control and the 7472insC mutant cell lines was amplified by PCR using primers FR31 and FR32 as previously [2]. From these fragments, a sequence between np 7585 and 7446, containing tRNA$^{Ser(UCN)}$ plus a 71 bp 5′-leader, was amplified using primer pair SP6L1 (<u>ATTTAGGTGACACTATAGAATAC</u>***GAATTC***TAAGATATATAGGATTTAGCCTATAAT) and SerCCA1 (<u>TGG</u>CAAAAAAGGAAGGAATCGAACC), all sequences shown 5′–3′. These primers introduced an SP6 promoter sequence (underlined), an *Eco*RI restriction site (bold italics, not used here in cloning, but introduced so as to reproduce exactly the substrate used by Puranam and Attardi), and a 3′ terminal CCA (double underlined). To avoid preparative PCR using primers of very different melting temperatures, we reamplified this fragment using primers SP6S1 (<u>ATTTAGGTGACACTATAGAATAC</u>***GAA***) and SerCCA1 and *Pfu* DNA polymerase to create blunt ends. The final products (SP6L-CCA-wt and SP6L-CCA-mut) were cloned and their structure was verified by sequencing. Plasmids containing these inserts were used as templates to create the final PCR products for in vitro transcription (using primer pair SP6S1/SerCCA1 and *Pfu* DNA polymerase), as well as to generate the additional wild-type and 7472insC mutant pre-tRNA$^{Ser(UCN)}$ substrates containing both a 5′-leader and a 3′-trailer. The 25 bp trailer sequence (bold, underlined) was introduced by primer Trail31 (**<u>AGAACCCGTATACATAAAATCTAGA</u>**CAAAAAAGGAAGGAATCGAACCC), used for PCR in combination with primer SP6S1. To create constructs with the specifically misprocessed 3′-end UCCA (bold, double-underlined) primer SerTCCA (**<u>TGGA</u>**CAAAAAAGGAAGGAATCGAAC) was used in combination with SP6S1. PCR conditions to create the

various constructs, as well as the final templates for in vitro transcription, were: 92 °C, 2 min; 30 cycles of (94 °C, 30 s, 55 °C, 30 s, and 72 °C, 30 s); and final extension 72 °C, 5 min. All such templates were gel-purified before use. Constructs for synthesis of pre-tRNA$^{Ser(UCN)}$ substrates with mature 5′-ends were as described previously [17].

RNA processing enzymes. RNase P was partially purified from HeLa cells as described previously [26]. Puranam and Attardi [15] reported that the mitochondrial RNase P from HeLa cells has the same properties, and contains the same catalytic RNA as the nuclear enzyme. Although this has been questioned [27], we believe that Attardi and Puranam in their response [27] provide compelling reasons for accepting their findings. We therefore used the enzyme prepared from whole cell extracts, under reaction conditions previously optimized for the 5′ processing of mitochondrial pre-tRNA$^{Ser(UCN)}$ [15,28]. Mitochondrial tRNA Z (tRNA 3′-processing endonuclease) was partially purified from HeLa cell mitoplasts fractionated by DEAE–Sepharose chromatography as previously [17].

In vitro RNA processing assays. Pre-tRNA$^{Ser(UCN)}$ substrates with mature 5′-ends were synthesized as described previously [17]. For other substrates, template PCR products were transcribed in vitro with SP6 RNA polymerase (MBI Fermentas) in the presence of [α-^{32}P]CTP (Amersham Biosciences, 3000 Ci/mmol) using manufacturer's recommended conditions. Template DNA was removed by addition of 1 U RNase-free DNAse (Roche) per μg of DNA template, with incubation at 37 °C for 15 min, followed by acid phenol extraction, chloroform extraction, and ethanol precipitation in the presence of glycogen carrier. The substrates were resuspended in DEPC-treated water and purified on 5% polyacrylamide-7M urea preparative gels, and re-folded in reaction buffer (see below) by heating for 2 min at 82 °C, then cooling for 15 min at room temperature. The 5′-processing assay was essentially as described by Doersen et al. [28]: approximately 0.01–0.02 pmol of the substrate was incubated in 200 μl of the reaction buffer (20 mM Tris, pH 7.9, 55 mM KCl, 5 mM MgCl$_2$, and 0.1 mM EDTA) plus 20 u RNasin and partially purified RNase P at various dilutions (see Figure legends) for 1 h at 37 °C. Reactions were terminated by the addition of an equal volume of stop buffer (0.2 mg/ml pronase, 0.5% SDS, 10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM EDTA) and incubation for 15 min at room temperature. Products were phenol extracted, ethanol precipitated in the presence of glycogen or carrier (yeast) tRNA, and analysed on 5% polyacrylamide-7M urea gels. The termini of the reaction products were analysed by gel extraction followed by circularization, RT-PCR, cloning, and sequencing, as described above for the in vivo analysis of tRNA$^{Ser(UCN)}$ ends. The 3′-processing reactions were performed in 20 μl of reaction buffer [17] plus partially purified mitochondrial tRNase Z at various dilutions (see Figure legends), for 30 min or 1 h at 37 °C. The reactions were stopped and the products were recovered and analysed as described above. Negative control reactions were performed in parallel, under the same conditions but without RNase P or tRNAse Z.

Quantitation of relative processing efficiency. To estimate the relative efficiency of processing of mutant and wild-type substrates, they were mixed in equal proportions prior to reaction. Reactions were carried out using different amounts of enzyme, and the extents of reaction were determined densitometrically from autoradiographs. Aliquots of the gel-extracted products and remaining unreacted substrates were reverse-transcribed using primer cser5 and then amplified with primers cser5 and cser6. The ratio of mutant to wild-type material was quantitated in all fractions by fluorescent primer extension (minisequencing), using primers cser3 and cser4 as previously [13]. The relative efficiencies of processing under different conditions were determined as the ratio of mutant to wild-type product divided by the ratio of mutant to wild-type material in the remaining substrate in each reaction.

Results

Misprocessing of pre-tRNA$^{Ser(UCN)}$ in vivo in 7472insC cybrid cells

To analyse the products of pre-tRNA$^{Ser(UCN)}$ processing in vivo from 7472insC mutant cells we extracted and deacylated total RNA from 143B osteosarcoma cybrid cells carrying 100 or 0% mutant mtDNA. After circularizing short RNAs by RNA ligase, tRNA$^{Ser(UCN)}$ was amplified using gene-specific primers, as shown in Fig. 1. RT-PCR products were cloned and sequenced, yielding the terminal sequences indicated in Table 1 and Fig. 1. RNA was analysed from cells grown on standard medium, as well as from cells treated with ethidium bromide (EtBr) for 2 days and then allowed to recover. Our reasoning for this was that any mis-processing products might be turned over rapidly, hence could be present only at low abundance, but might be detected more easily in cells recovering from mtDNA and RNA depletion. However, the frequencies of aberrantly processed products in EtBr-treated and untreated cells were similar, therefore the data are simply pooled in Table 1.

Over 11% of all tRNA$^{Ser(UCN)}$ molecules from 7472insC mutant cells analysed by this method had incorrect 5′ and/or 3′ termini (330 clones analysed, 158 being from EtBr-untreated cells). This may be an under-estimate of the frequency of misprocessing in vivo, because of the possibility that misprocessed molecules are less efficiently circularized. The most common 5′ processing error found (∼15% of all misprocessed molecules) was addition of one extra 5′ terminal nucleotide (U). The spectrum of 3′ processing errors was more diverse, including the addition of one or more 3′ terminal nucleotides (U, UC, UCU or UCUA), loss of the discriminator base (G) or more extensive 3′ truncation, and even the presence of one or more non-templated nucleotides in place of CCA. Some molecules with additional 3′ nucleotides also lacked, or partially lacked, CCA, so that in a few cases it was not possible to determine with certainty whether a terminal U had come from the 5′ or 3′ end, or whether a terminal C had been created by misprocessing (generating 3′ UC beyond the discriminator base) or by addition of C by tRNA-3′ nucleotidyl transferase to a misprocessed 3′ end terminating in an additional U. In Table 1 we indicate as 5′ misprocessed only those molecules where the provenance of the additional nucleotide(s) was unambiguously from the 5′ end. We indicate as 3′ misprocessed only those that could not have arisen merely from truncation of CCA.

Many molecules clearly exhibited processing errors at both ends. Amongst 192 molecules analysed from control cybrid cells (0% 7472insC, mtDNA from the same patient, 96 of which were from EtBr-untreated cells), only two cases of tRNA$^{Ser(UCN)}$ misprocessing were
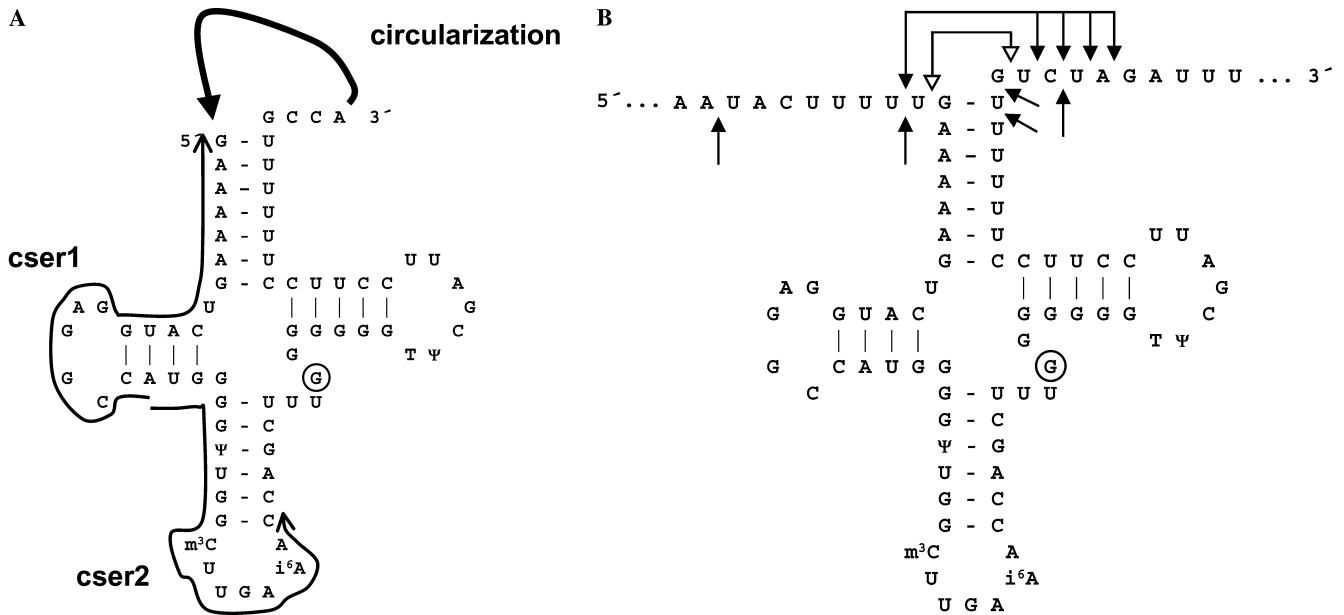
Fig. 1. Spectrum of tRNA$^{Ser(UCN)}$ processing errors detected in vivo in cells carrying 100% 7472insC mutant mtDNA. The sequence of tRNA$^{Ser(UCN)}$ is as determined previously [13], with the additional G templated by the mutation denoted by a circle. (A) Strategy for circularization and RT-PCR of tRNA$^{Ser(UCN)}$. Oligonucleotides cser1 and cser2 are shown alongside the portions of the tRNA to which they correspond (cser2) or are complementary (cser1), the arrowheads depicting their orientation (5′–3′). (B) Physiologically correct processing sites are shown by open arrowheads, with the incorrect processing sites that were detected shown by filled arrowheads. The horizontal line denotes the different incorrect 3′ processing sites that were detected in combination with an additional 5′ terminal U. Some 3′ misprocessed molecules also lacked or partially lacked CCA (see Table 1) and some of these also had inserted, non-templated nucleotides.

Table 1
tRNA$^{Ser(UCN)}$ molecules with various termini detected in vivo[a]

Class	Wild-type cells	7472insC mutant cells
Total number of clones analysed	192	330
Correct termini (%)	96.9	86.9
5′ misprocessed (total)	<1[c]	6.7
5′ defect only	0	4.2
5′ and 3′ defects	<1[c]	2.5
3′ misprocessed (total)	<1[c]	6.2
3′ defect ± CCA[b]	<1[c]	4.5
3′ defect + inserted nts ± CCA	0	1.7
CCA defect only[b]	2.1	2.0

[a] Approximately equal numbers of clones were analysed from cells grown in normal medium and from cells treated transiently with EtBr, both from wild-type (96 + 96) and mutant cells (158 + 172). No significant differences in the frequency of misprocessed forms of tRNA$^{Ser(UCN)}$ were found under the two conditions, therefore data are pooled.
[b] CCA wholly or partly missing.
[c] Based on single cases.

found, one misprocessed at both ends, the other only at the 3′ end. Lack of a complete CCA was found in both mutant and control cells at the same frequency (~2%), and is assumed to represent either damaged or immature molecules. The data strongly suggest that the mutation affects both the efficiency and specificity of tRNA$^{Ser(UCN)}$ processing at both termini.

7472insC impairs both 5′ and 3′ processing of pre-tRNA$^{Ser(UCN)}$ in vitro

In order to determine more precisely the effects of the mutation on pre-tRNA$^{Ser(UCN)}$ processing, we designed constructs for in vitro transcription (Fig. 2), which were used to create labelled substrates with various 5′ and/or 3′ ends, plus or minus the 7472insC mutation. These substrates were processed in vitro using partially purified extracts of RNase P or tRNase Z, and the products were analysed by a combination of denaturing polyacrylamide gel electrophoresis/autoradiography and circularization plus cloning and sequencing, as used for the in vivo analysis.

We initially confirmed that HeLa cell RNase P was able correctly to process pre-tRNA$^{Ser(UCN)}$ substrates with an extended 5′ leader, but already matured at the 3′ end, including CCA addition. Under conditions where the reaction went to completion, uniformly labeled mutant and wild-type substrates were correctly processed to a leader fragment of 83 nt, as well as heterogeneous, tRNA-sized products of approximately 72 nt (Fig. 2A). Circularization, RT-PCR cloning and sequencing of the tRNA-sized products confirmed that they had the correct 5′ ends (162/162 clones analysed, 79 from wild-type and 83 from mutant), but with variable trimming at the 3′ end (0–5 nt), accounting for the heterogeneity seen in the gel. The 3′ heterogeneity is presumed to have
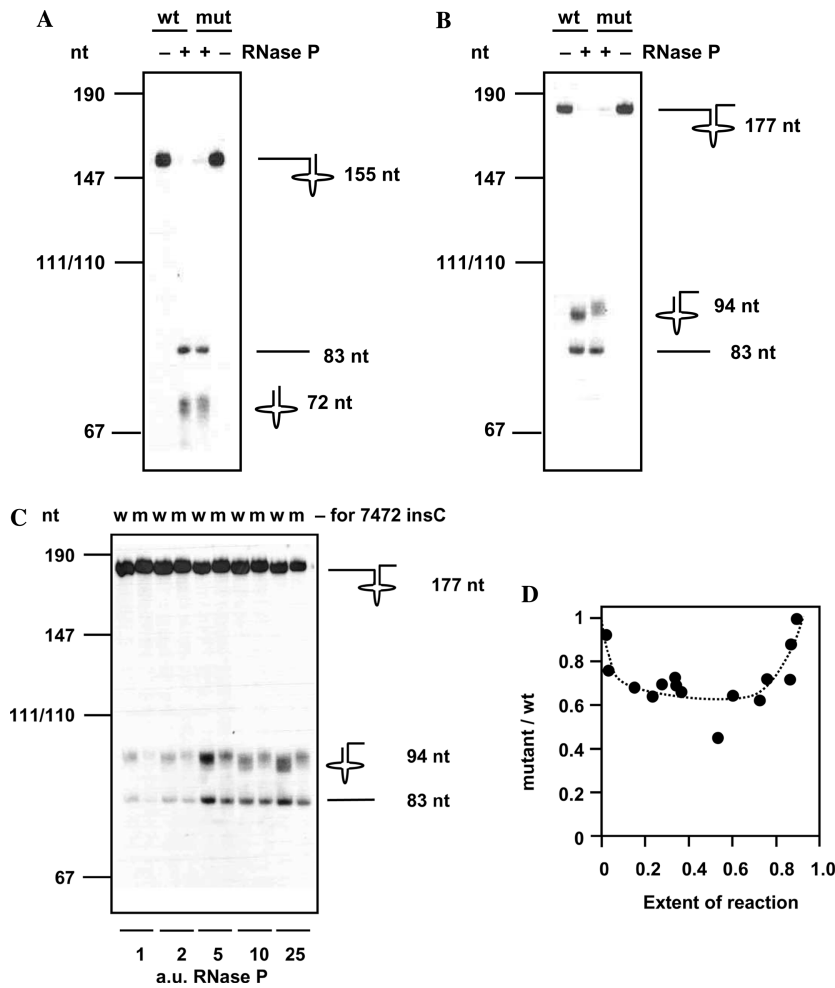
Fig. 2. 5′ processing of pre-tRNA$^{Ser(UCN)}$ substrates in vitro. (A) Uniformly labeled wild-type and 7472insC mutant substrates with an 83 nt 5′ leader, already bearing correct 3′ ends including CCA, 5′ processed to completion with (+) or without (−) excess HeLa cell RNase P. Positions of DNA size markers shown on left, cartoons of substrates and products on right. Note the size heterogeneity of mature tRNA products, due to 3′ end trimming in the extract. (B) Uniformly labeled wild-type and 7472insC mutant substrates with an 83 nt 5′ leader and a 25 nt 3′ trailer, 5′ processed to completion with (+) or without (−) excess HeLa cell RNase P. (C) The same substrates as in (B), denoted as w (wild-type) and m (7472insC mutant), 5′ processed using increasing amounts (arbitrary units) of HeLa cell RNase P (via serial dilution of the enzyme). A similar result was obtained in three independent experiments. (D) Relative efficiency of 5′ processing of the same substrates as in (B,C), using increasing amounts of HeLa cell RNase P. The overall extent of reaction was determined densitometrically, in each case, from the ratio of products to substrate. The relative processing efficiencies were determined by RT-PCR and minisequencing, of excised product and remaining substrate bands, normalized against unprocessed substrate. The values plotted are averages of independent minisequencing analyses for the two strands. The dotted line, fitted by eye, indicates that the mutant substrate was processed at approximately 60% of the efficiency of the wild-type substrate under a wide concentration range.

resulted from 3′ exonuclease activity in the extract, but since the leader fragment was not trimmed, it may be a tRNA-specific activity, possibly related to the previously documented tRNA repair pathway [29]. Exonuclease activity was not detected in previous experiments where the same extracts were used to map RNase P cleavage positions in HCV [26]. Possible 3′ heterogeneity in the in vitro transcription product itself may also contribute.

In a similar reaction, 5′ processing of substrates containing an extensive 3′ trailer as well as a 5′ leader yielded the same excised leader fragment, plus the tRNA still attached to the 3′ trailer, again showing 3′ heteroge-neity (Fig. 2B). Using lower amounts of enzyme (Figs. 2C, and D), i.e., under conditions where the reaction did not go to completion, the mutant substrate was also reproducibly processed to a lesser extent than the wild-type (Fig. 2C). In order to quantitate this, we mixed equal amounts of the two substrates and processed them in single reactions, using different amounts of enzyme. We determined the overall extent of reaction in each case, by comparing the amounts of unprocessed sub-strate and of the reaction products densitometrically. The relative efficiencies of processing of mutant and wild-type substrates were then determined by quantitating

the relative amounts of mutant and wild-type molecules in gel-excised substrate and product bands by RT-PCR minisequencing and normalizing for the ratio of the two in the unprocessed starting material at $t = 0$ (Fig. 2D). Across a wide range of extents of reaction, the mutant substrate was processed to approximately 60% of the extent of the wild-type substrate, the only consistent deviations from this being seen when the reaction was near completion (saturating amounts of enzyme) or where hardly any processing was observed, when background signals from non-specific scission of the probe become significant.

We tested the effects of the mutation on 3′ processing of two different types of substrate, using partially purified extracts of mitochondrial tRNase Z as used in previous studies of the A7445G mutation [17]. The first substrate tested was similar to the one used earlier in studies of the A7445G mutation, i.e., tRNA$^{Ser(UCN)}$ plus a 3′ trailer, but with the 5′ end already correctly processed (Fig. 3A). 7472insC appeared to have no effect on the reaction using this substrate. However, the mutation substantially affected 3′ processing of a substrate containing both 5′ leader and 3′ trailer segments (Fig. 3B). The identity of the major product resulting from processing of the wild-type substrate was verified by circularization, RT-PCR, cloning, and sequencing. The mutant substrate was processed much less efficiently, with incorrect or non-specific products correspondingly more prominent. The amount of non-specific product makes it difficult to derive a reliable estimate of the relative efficiencies of 3′ processing of wild-type and mutant substrates, whether by densitometry or RT-PCR minisequencing, but it is clearly several-fold.

3′ misprocessing of 7472insC pre-tRNA$^{Ser(UCN)}$ impairs its 5′ processing in vitro

The above findings indicate that 3′ processing of the 7472insC mutant substrate is greatly impaired if 5′ processing has not already taken place, and that non-specific products predominate under these conditions. Having detected a prominent class of tRNA$^{Ser(UCN)}$ molecules misprocessed at both termini, we reasoned that misprocessing at the 3′ end may further impair 5′ processing and promote the specific type of 5′ misprocessing that we detected in vivo, i.e., the retention of an additional 5′ terminal U. To test this, we created a deliberately 3′ misprocessed substrate, terminating in (discriminator G)-UCCA instead of (discriminator G)-CCA, and processed it in vitro using RNase P (Fig. 4A). As controls we tested in parallel a substrate wild-type at np 7472 ending also 3′ misprocessed in the same way, as well as a completely wild-type substrate terminating in (discriminator G)-CCA. Both of the 3′ misprocessed products were processed in vitro by RNase P, although the substrate also containing the 7472insC mutation was processed less efficiently than wild-type, especially in the presence of low amounts of the enzyme (Fig. 4B).

The reaction products were further analysed by circularization, RT-PCR, cloning, and sequencing. Because of the presence, in some substrate molecules, of nontemplated 3′ nucleotides (most commonly A, but also U), we were unable to reliably quantitate the amount of specific 5′ misprocessing of the artificially 3′ misprocessed substrates ending (discriminator G)-UCCA. However, several cases were found where an additional
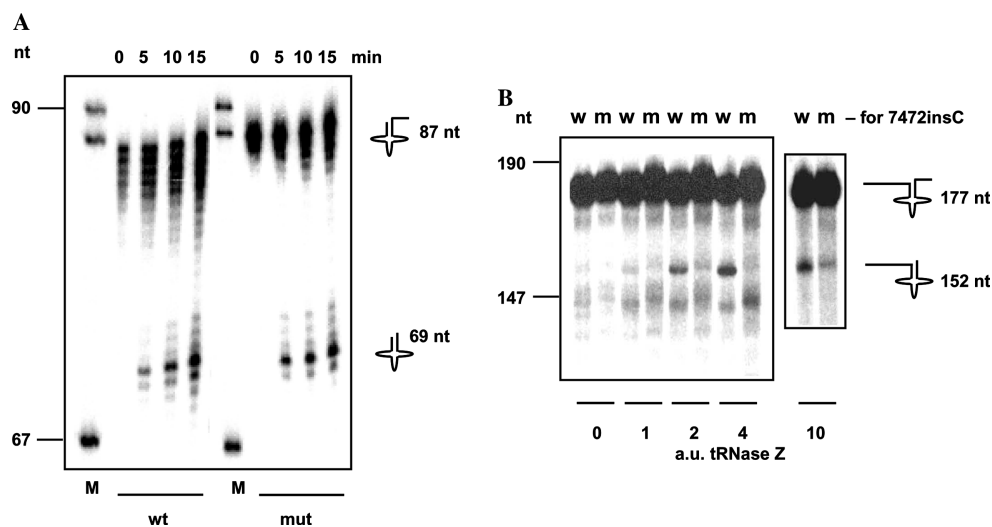

Fig. 3. 3′ processing of pre-tRNA$^{Ser(UCN)}$ substrates in vitro. (A) 5′ end-labeled wild-type and 7472insC mutant substrates with an 18 nt 3′ trailer, already bearing correct 5′ ends, 3′ processed for increasing times with partially purified mitochondrial tRNase Z (tRNA 3′ processing endonuclease) from HeLa cells. Positions of DNA size markers shown on left, cartoons of substrates and products on right. (B) Uniformly labeled wild-type (w) and 7472insC mutant (m) substrates with an 83 nt 5′ leader and a 25 nt 3′ trailer, 3′ processed using increasing amounts (arbitrary units) of tRNase Z (via serial dilution of the enzyme). The identity of the major product band was verified by gel excision and sequencing.
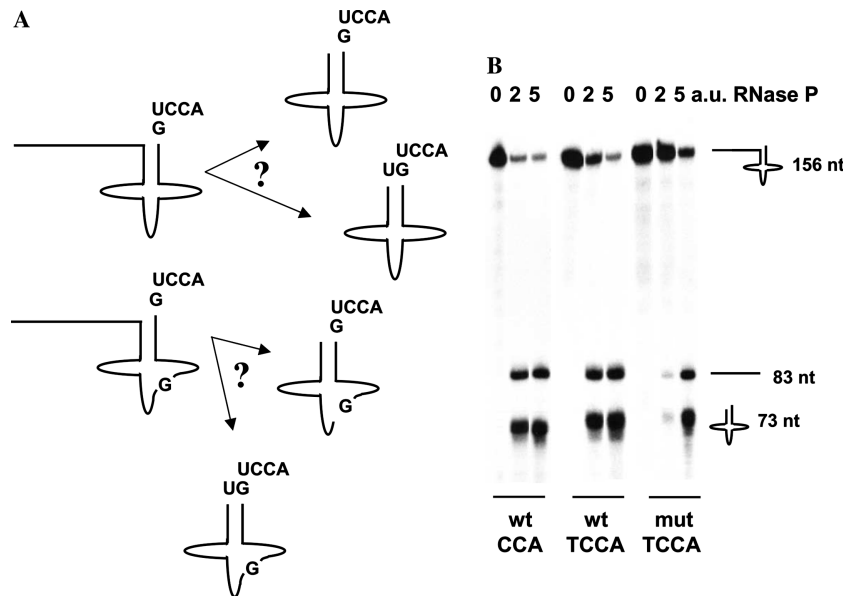
Fig. 4. 5′ processing of 3′ misprocessed pre-tRNA$^{Ser(UCN)}$ substrates in vitro. (A) Cartoons of the 3′ misprocessed substrates tested, carrying an additional 3′ U between the discriminator residue (G) and CCA, plus or minus the additional G templated by the 7472insC mutation. The predicted 'correct' and 5′ misprocessed products are as shown. (B) Uniformly labeled wild-type and 7472insC mutant substrates with an 83 nt 5′ leader, plus the 3′ misprocessed terminus (discriminator G)-UCCA or the correct 3′ end (discriminator G)-CCA. Positions of DNA size markers shown on left, cartoons of substrates and products on right.

U was present, which was inferred to have derived from the 5′ end, since the CCA end had been wholly or partially truncated. Such clones were not detected using wild-type substrates.

Discussion

The 7472insC mutation impairs pre-tRNA$^{Ser(UCN)}$ processing

Previous analyses showed that the 7472insC mutation decreases the rate of tRNA$^{Ser(UCN)}$ synthesis post-transcriptionally, without affecting its turnover or pattern of base-modification [13]. Using a combined in vivo and in vitro approach, we here demonstrate that both 5′ and 3′ end maturation are affected. The efficiency of CCA addition per se does not appear to be affected directly, although we cannot exclude that some rare cases of non-templated 3′ nucleotides such as we observed arise from errors in CCA addition. Our previous study indicated that the mutation, which adds one nucleotide to the extra loop of the tRNA, does not produce a gross structural distortion of the tRNA such as can be detected, e.g., on the basis of nuclease sensitivity [13]. However, it would appear to be sufficient to alter the interaction between at least the tRNA$^{Ser(UCN)}$ precursor(s) and both RNase P and tRNase Z, so as to make processing at both termini considerably less efficient.

Based on the in vitro results, the impairment of 3′ processing seems to be quantitatively more significant.

However, the effects at each terminus appear to be contingent on what happens at the other. Processing at the 3′ end of substrates in which the 5′ end is already processed is hardly affected at all, whereas if the 5′ end remains unprocessed the mutation entrains a significantly reduced rate of 3′ processing. Conversely, the effect of the mutation on the rate of 5′ processing is not contingent on whether the 3′ end is already processed. However, specific misprocessing at the 3′ end promotes misprocessing at the 5′ end. The mutation thus affects processing at both termini and does so in both a quantitative and qualitative manner. This 'contingent misprocessing' represents a novel pathological mechanism for a mitochondrial tRNA mutation.

Determinants of 5′ and 3′ pre-tRNA processing

Enzymes involved in tRNA end maturation must have, by definition, both a broad specificity (in order to process at least 22 and usually over 30 different substrates), and accurate recognition of determinants that specify precise cleavage. In the case of both RNase P and tRNase Z in animal cells these potentially conflicting requirements are further exacerbated by the fact that single, or closely related, enzymes are required to process two distinct sets of tRNA in the nucleus and mitochondria, many of the latter lacking some canonical features of tRNAs, and with at least several examples per species of structurally unusual forms, notably the seryl tRNAs. Processing of mitochondrial tRNA$^{Ser(UCN)}$ might be especially sensitive to mutations, not only

because of its already aberrant structure (6 bp anticodon stem, short D-loop), but because it is a rare example of a 'stranded tRNA' in the mitochondrial genome, with the nearest L-strand encoded RNAs located 7.2 kb upstream and 1.6 kb downstream of its termini. This means that the precursor transcript is kilobases in length.

A frequent type of 3′ misprocessing which we identified in vivo in 7472insC mutant cells was the retention of an additional 3′ terminal U beyond the discriminator G. This may be the result of 'stuttering' by mammalian tRNase Z, which can cleave at various positions around the discriminator, depending on the precise length or structure of the acceptor stem and T-arm [30]. Cleavage 1 nt after the discriminator, leaving the additional 3′ terminal U, might favour the formation of an extra U–G base-pair between the discriminator base and the 5′-1 U of the precursor. We propose that this base-pairing promotes low-level misprocessing by RNase P on the 5′ side of the −1 U, as was observed both in vivo and in vitro. In bacteria, RNase P appears to have a strong preference for U at the −1 position [31,32], although this cannot apply strictly in human mitochondria, where slightly more than half of all tRNAs have a different nucleotide at −1. However, the −2 nucleotide in tRNA$^{Ser(UCN)}$ is also U; therefore, if a −1 U substrate is preferred, 5′ misprocessing following 3′ misprocessing would not be disfavoured.

The effects of the 7472insC mutation on RNase P are not easily predictable. In general, the 'top half' of tRNAs (acceptor stem, T-loop, and T-stem) appears to be the most crucial determinant of specificity [33,34], although mostly this relies on inferences from studies of the bacterial enzyme. In vitro, both 7472insC and the 3′ misprocessed end (discriminator G)-UCCA seemed to affect 5′ processing more strongly when the amount of enzyme was low, suggesting that the aberrant structures have lower affinity for the enzyme, presumably as a result of altered relative spacing of the top of the acceptor stem and the T-stem, or altered overall length of the helical region [34]. The additional G templated by 7472insC could extend the extra loop or T-loop by 1 nt, or the T-stem by 1 bp (reducing the T-loop by 1 nt or shifting the register of the acceptor stem). Previous studies of nuclease sensitivity [13] did not resolve this issue, since the entire region was inaccessible to nucleases.

In both plants [35] and mammalian cells [30], the acceptor stem length appears to be a critical determinant for tRNAse Z, although the minimal substrate also includes the T-arm [30,36]. A previous study of the human mitochondrial enzyme [17] revealed that a C immediately following the tRNA$^{Ser(UCN)}$ discriminator, as created by the A7445G mutation, constitutes an anti-determinant. In the present study, the 7472insC mutant pre-tRNA$^{Ser(UCN)}$ was a poor substrate for mitochon-

drial tRNase Z in vitro, unless it was pre-processed at the 5′ end. This suggests the possibility that the mutation might favour or enforce an abnormal structure in the unprocessed precursor that is lost when the 5′ leader is removed. An obvious possibility would involve incorrect base-pairing between the extended G tract created by the mutation and the run of 14 consecutive pyrimidines immediately upstream of the discriminator. A wide spectrum of mitochondrial tRNA mutations is now known to interfere with 3′ pre-tRNA processing, including A7445G (tRNA$^{Ser(UCN)}$, trailer, [17]), tRNA$^{Leu(UUR)}$ mutations [37] A3243G (D-loop), A3302G, and C3303T (acceptor stem), and tRNA^{Ile} mutations [38] A4269G (acceptor stem), A4295G (anticodon loop), G4309A (T-stem), and A4317G (T-loop). What they have in common is unclear, but tRNase Z would appear to be sensitive to many different structural perturbations of mitochondrial pre-tRNA substrates.

The severity of the 7472insC processing defect depends on the processing pathway used in vivo

Previous authors have suggested that 5′ processing of mitochondrial tRNAs generally precedes 3′ processing [21], although this assertion is based on isolated examples and few cell-types, plus analogy with other systems. In contrast, the processing pathway for mitochondrial tRNA$^{Leu(UUR)}$ varies between muscle (as well as some other solid tissues, L.A. Bindoff, personal communication), where a 5′ processed but 3′ unprocessed precursor is detected, and cultured cells, where a 3′ processed but 5′ unprocessed precursor is detected [22], implying an opposite order of processing. Measurements of the amount of RNase P in human mitochondria indicate that, in cultured cells, it is present at very low abundance [15], consistent with this finding.

In previous studies we and others failed to detect any discrete precursor of pre-tRNA$^{Ser(UCN)}$ in cultured cells [12], concluding that any light-strand processing intermediates are highly heterogeneous, and that processing may be iterative. Furthermore, in a previous study of the A7445G mutation, which affects the 3′ processing site of pre-tRNA$^{Ser(UCN)}$, it was shown that the mutation completely blocks tRNase Z action in vitro, whereas the level of tRNA$^{Ser(UCN)}$ in vivo is reduced only by approximately 65–70% [17], indicating that an alternative pathway for 3′ end formation must operate in vivo. This may involve one or more unidentified endonucleases and/or 3′ trimming by exonuclease(s), as in bacteria. Since the specificities of both RNase P and tRNase Z are of necessity broad, the possible cloverleaf-like structure adopted by the antisense transcript of tRNA^{Asp}, which lies adjacent to the 5′ end of pre-tRNA$^{Ser(UCN)}$, may facilitate processing by tRNase Z immediately upstream of pre-tRNA$^{Ser(UCN)}$ (Fig. 5A), perhaps stimulated by RNase P processing on the other side of anti-
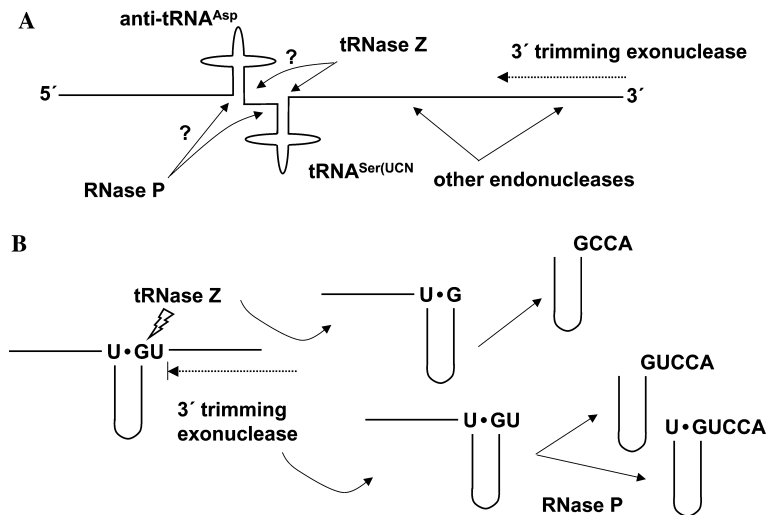
Fig. 5. Possible processing pathways of mitochondrial pre-tRNA$^{Ser(UCN)}$. (A) RNase P cuts at the 5′ end of tRNA$^{Ser(UCN)}$, but may also cut at the 5′ end of the antisense transcript of tRNA^{Asp}. Similarly, tRNase Z cuts at the 3′ end of tRNA$^{Ser(UCN)}$, but may also cut at the 3′ end of the antisense transcript of tRNA^{Asp}, which is close to the 5′ end of tRNA$^{Ser(UCN)}$. Other nucleases may also be involved in the formation of the 3′ end of tRNA$^{Ser(UCN)}$ via alternative pathways. (B) Incorrect 3′ end maturation of tRNA$^{Ser(UCN)}$ is proposed to entrain a significant chance of 5′ misprocessing, incorporating an additional U–G base-pair in the aminoacyl stem of the tRNA.

tRNA^{Asp}. Although such processing events may be minor, and were not detected in vitro in the present study, it cannot be excluded that they contribute to the natural processing of the light-strand transcript in some tissues in vivo.

The effect of the 7472insC mutation on tRNA$^{Ser(UCN)}$ biosynthesis in vivo will therefore depend on the precise RNA processing pathway used in specific tissues or individuals, thus contributing to the tissue-specificity and variability of the resulting disorder. Where mitochondrial RNase P is in excess, or where the presence of RNA binding proteins or other factors enforces the primacy of 5′ processing (e.g., in muscle), the effect of the 7472insC mutation is predicted to be minor, based on our in vitro results. There would be a small drop in the rate of 5′ processing, but the 5′ processed intermediate should be processed almost normally by tRNase Z, such that the final steady-state level of tRNA$^{Ser(UCN)}$ would approach that in wild-type cells. Conversely, in cases where 3′ processing is the required first step, the 7472insC mutation is predicted to cause a significant drop in the rate of processing by tRNase Z, increasing the chance that 3′ end formation will follow an alternate pathway, and that the 3′ end created will not be correct. Exonucleolytic 3′ trimming may not efficiently remove the 3′ terminal U adjacent to the discriminator, since the discriminator G can base-pair with the U residue still present upstream of the as-yet unprocessed 5′ end in the predicted intermediate (Fig. 5B). Misprocessing of this type, followed by CCA addition, would, based on our in vitro results (Fig. 4), create a mutant substrate that would be poorly processed by RNase P, with an increased chance of 5′ mis-processing to retain the additional U, as also seen frequently in vivo.

The spectrum of aberrant 3′ ends seen in mutant cells in vivo, including some with non-templated nucleotides, suggests that where tRNase Z cleavage is not efficient, alternate pathways of 3′ end formation must include the repair mechanism previously documented [29]. Aberrant termini may result from the inherent error-proneness of tRNA repair, coupled with the fact that a much higher proportion of 3′ ends are generated in vivo by these pathways than in control cells. Alternatively, the 7472insC mutation might further impair the accuracy of tRNA repair. The aminoacylation and translational properties of the various misprocessed forms are unknown, but mischarging, leading to specific misincorporation of amino acids other than serine at UCN codons, may be one possible outcome.

The identification of the major endoribonucleases involved in mitochondrial pre-tRNA processing [15,16] now allows some of these issues to be addressed, by analyzing the expression and extent of mitochondrial localization of these two enzymes in different tissues, including those, e.g., cochlea, cerebellum, most relevant to 7472insC disease.

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