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Evidence for Import of a Lysyl-tRNA into Marsupial Mitochondria

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The mitochondrial tRNA gene for lysine was analyzed in 11 different marsupial mammals. Whereas its location is conserved when compared with other vertebrate mitochondrial genomes, its primary sequence and inferred secondary structure are highly unusual and variable. For example, eight species lack the expected anticodon. Because the corresponding transcripts are not altered by any RNA-editing mechanism, the lysyl-tRNA gene seems to represent a mitochondrial pseudogene. Purification of marsupial mitochondria and in vitro aminoacylation of isolated tRNAs with lysine, followed by analysis of aminoacylated tRNAs, show that a nuclear-encoded $tRNA^{Lys}$ is associated with marsupial mitochondria. We conclude that a functional $tRNA^{Lys}$ encoded in the nuclear genome is imported into mitochondria in marsupials. Thus, tRNA import is not restricted to plant, yeast, and protozoan mitochondria but also occurs also in mammals.

INTRODUCTION

Generally, vertebrate mitochondria encode all structural RNAs necessary for autonomous protein synthesis (Anderson *et al.*, 1981). These RNAs include two rRNAs and a set of 22 tRNAs, which are sufficient to decipher the mitochondrial genetic code. However, import of nuclear-encoded tRNAs has been reported for plant, yeast, and protozoan mitochondria, whereas import of RNA into mammalian mitochondria has been proposed only for other structural RNAs (Yoshionari *et al.*, 1994). By contrast, most mitochondrial proteins are encoded in the nucleus and subsequently imported into mitochondria, and much is known about the mechanisms and signals that are involved in this process (Neupert, 1997).

In the last years, in vivo and in vitro tRNA import systems have been developed in plants (Small *et al.*, 1992), trypanosomatids (Adhya *et al.*, 1997), and yeast (Tarassov and Entelis, 1992), so that direct evidence for tRNA import in mitochondria is available. The most studied nuclear-encoded RNA proposed to be imported into mammalian mitochondria is the RNA subunit of the mitochondrial endoribonuclease (RNase MRP), which is involved in RNA primer processing during mtDNA replication (Chang and Clayton, 1987). Although its mitochondrial localization has been questioned (Kiss and Filipowicz, 1992), in situ hybridization data support the dual location of RNase MRP RNA in mouse cardiomycetes (Li *et al.*, 1994). An additional candidate for RNA import is the RNA subunit of RNase P, an enzyme involved in 5′ processing of tRNAs. Finally, an association of 5S rRNA with mammalian mitochondria was reported (Yoshionari *et al.*, 1994; Magalhaes *et al.*, 1998). However, the function of cytosolic 5S rRNA in mitochondria remains unclear.

In this study, the mitochondria-encoded tRNA gene for lysine in 11 marsupials was analyzed, because primary sequence and presumed secondary structure of the $tRNA^{Lys}$ transcript in the wallaroo indicate that this endogenous tRNA gene does not encode a functional lysyl-tRNA (Janke *et al.*, 1997). Furthermore, the tRNA transcripts are not altered posttranscriptionally to resemble more conventional $tRNA^{Lys}$ structures. After subcellular fractionation and sequential purification of marsupial mitochondria the only mitochondrial tRNA that can be aminoacylated with lysine in the presence of a marsupial mitochondrial S100 extract is a nuclear-encoded lysyl-tRNA. A likely explanation for these results is that tRNA import takes place.

MATERIALS AND METHODS

Animal Tissue

Living North American opossums (*Didelphis virginiana*) were purchased from Roberts Serpents Inc. (Riverview, FL) and small opossums (*Monodelphis domestica*) were purchased from the university hospital (Hamburg-Eppendorf, Germany). DNA samples from *Isoodon macrourus* (bandicot), *Smithopsis crassicaudata* (marsupial mouse), and *Phascolarctos cinereus* (koala bear) were obtained from the museum of vertebrate zoology of the University of California, Berkeley, CA. Fresh or frozen liver material from *Macropus agilis* (flink wallaby), *Macropus robustus* (mountain kangaroo), *Macropus giganteus* (gray kangaroo), *Macropus rufus* (red giant kangaroo), and *Macropus eugenii* (tammar wallaby) were donated by Tierpark Hellabrunn (Munich, Germany). Kidney fibroblast cells from *Potorous*

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tridactylus (small kangaroo) were ordered from the American type tissue culture collection. Mouse tissue was obtained from the Zoological Institute (Munich, Germany).

DNA and RNA Preparation

Total cellular DNA was isolated from tissues (Sambrook *et al.*, 1989). Cellular RNA was prepared by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987) from fresh or frozen material. High molecular weight RNA was precipitated by the addition of NaCl to a final concentration of 1 M at 4°C. After centrifugation, the small RNA fraction in the supernatant was ethanol precipitated and redissolved in water for further analysis.

Oligonucleotides

Oligonucleotides for the amplification of the mitochondrial lysine-tRNA gene in different marsupials were as follows: flank-CoII (5′-CTA AAA TAT TTC GAA AAA-3′) and flank-ATP8 (5′-TAA TGT CTG GTT TAT TAT TT-3′).

Primers for cDNA first-strand synthesis (designated 1) and subsequent polymerase chain reaction (PCR) amplification (designated 2) of mitochondrial lysyl-tRNA from opossum (*D. virginiana*, Dv), small kangaroo (*P. tridactylis*, Pt), red giant kangaroo (*M. rufus*, Mru), and mountain kangaroo (*M. robustus*, Mro): Dv1 (5′-TAT TTT GAG GAG TTT CT-3′); Dv2 (5′-TTT TTG AGT TAT TTA TA-3′); Pt1 (5′-GTT TTG AGG GCT TTT-3′); Pt2 (5′-CTT TGA GAA ATT ATT A-3′); Mr1 (5′-GTT TTG AGG GTT TAT-3′); Mr2 (5′-TTT TGA GAA ACC CA-3′); for Mru and Mro the same primers were used. Primer Mr1 has three and primer Mr2 has one mismatch to *M. rufus* lysyl-tRNA. The annealing temperature for the PCR amplification was decreased correspondingly.

Primers for cDNA synthesis and PCR amplifications of *D. virginiana* mitochondrial tRNAs glutamic acid (glu), glutamine (gln), asparagine (asn), and glycine (gly) were the following: glu1 (5′-TTT TTA TAT GGA CTC TAA C-3′), glu2 (5′-TTT TTG TAG TTG AAA TAC-3′); gln1 (5′-AGA ACA ATA GGC TTG AAC-3′), gln2 (5′-GAA TGT GGT GTA AAG GAA-3′); asn1 (5′-CTA AAC TGG AGG GTA TT-3′), asn2 (5′-TAA ATT GAA GCC GAA TG-3′); gly1 (5′-TGG TTT TCT CTG GTT T-3′), gly2 (5′-TTT TTC TAG TAT AAT TAG-3′).

Oligonucleotides for filter hybridizations: mt. K-op, recognizing *D. virginiana* mitochondrial $tRNA^{Lys}$ (5′-TTT TGA GGA GTT TCT TAG GTC-3′), mt.K-cons, recognizing conventional mitochondrial $tRNA^{Lys}$ (5′-CTT TAG CTT AAA AGG CCA-3′), 12S rRNA (5′-TGG GGT ATC TAA TCC CAG TTT-3′), cyt.L, recognizing cytoplasmic $tRNA^{Leu}$ (5′-CAG CGC CTT AGA CCG CTC-3′).

Oligonucleotides for cDNA synthesis and amplification of circularized tRNAs lysine (K) and aspartic acid (D) from *M. domestica* and human lysyl-tRNA: Kcirc1 (5′-AAG TAT AGA CTA ATT GC-3′), Kcirc2 (5′-GGT ATG CAA GAG ATA T-3′), Dcirc1 (5′-AGT TAT GTA ATT ATT TTA CTA A-3′), Dcirc2 (5′-GCC ATA GTT AAA TTA CAA G-3′), KHscirc1 (human) (5′-CTT TAA CTT AAA AGG TTA ATG CT-3′), KHscirc2 (human) (5′-ATT AAG AGA ACC AAC ACC TC-3′); amplification of tagged cDNA: pUC18 (5′-AAC AGC TAT GAC CAT G-3′).

cDNA Synthesis

Total cellular RNA (2 μg) or small RNA (0.5 μg) were incubated with 10 U of RNase-free DNase (Amersham Pharmacia Biotech, Piscataway, NJ) in 50 mM Tris/HCl, pH 8.3, 20 mM KCl, and 10 mM $MgCl_2$ at 37°C for 15 min. The DNase was inactivated then for 2 min at 100°C. Annealing of the first-strand primer (1 pmol) was performed in 20 μl of TE (10 mM Tris/HCl, and 2 mM EDTA, pH 8.0). After heating to 95°C for 3 min, the reaction was left at 37°C for 15 min and subsequently at room temperature for 10 min. First-strand cDNA synthesis was carried out in a total volume of 30 μl in 50 mM Tris/HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 20 mM deoxyribonucleotide triphosphates (dNTPs), and 5 U of avian myeloblastosis virus (AMV) reverse transcriptase (Stratagene, La Jolla, CA) at 37°C for 1 h. In control experiments, the RNA was treated with RNase A (0.1 mg/ml final concentration) for 15 min at 37°C before cDNA synthesis.

PCR and Sequencing

PCR amplifications of genomic DNA and cDNA contained 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mg bovine serum albumin/ml, 1 mM dNTPs, 1 pmol of each primer, and 20 U of *Taq* DNA polymerase/ml (Perkin Elmer-Cetus, Norwalk, CT) in a volume of 25 μl. The temperature profile for 40 PCR cycles consisted of 95°C for 1 min, 40- 50°C for 1 min and 72°C for 1 min.

PCR products were gel purified (GeneClean kit, Dianova, Hamburg, Germany) and directly sequenced by the dideoxy chain-terminating method (Sanger *et al.*, 1977). Oligonucleotides for sequencing were the same as for PCR amplification.

Because the dihydrouridine stem (DHU), TΨC, and variable loop nucleotides of mitochondrial tRNAs are the most variable regions, these regions were excluded from the alignment and computation.

Filter Hybridizations

For Southern blot hybridization total DNA from *D. virginiana* and mouse was digested with different restriction enzymes according to the supplier's instructions, phenol extracted, and ethanol precipitated. Restriction fragments were fractionated on a 1% agarose gel in a 40 mM Tris-acetate buffer and transferred to a nitrocellulose membrane (Sambrook *et al.*, 1989). For detection of the *D. virginiana* and mouse mitochondria-encoded lysyl-tRNA gene, [5′-^{32}P]γ-ATP-labeled oligonucleotides mt. K-op and mt. K-cons were hybridized to the membrane. Washes were performed under stringent conditions before exposure.

For dot blot hybridization of mitochondrial RNA, the samples were desiccated and resuspended in 2–4 μl of a denaturing solution (15% formaldehyde, 10× SSC). The samples were heated for 15 min at 60°C before spotting onto a Hybond N+ membrane (Amersham Pharmacia Biotech) that had been soaked for 10 min in 10× SSC. After air drying, the membrane was UV cross-linked for 1 min at 600 J and then baked for 2 h at 80°C. Prehybridization was carried out in 1 ml/cm^2 Rapid-hyb buffer (Amersham Pharmacia Biotech) for 1 h. For hybridization, the [5′-^{32}P]γ-ATP-radiolabeled oligonucleotide was added to the same solution and incubation was continued for another 3 h. The temperature for prehybridization and hybridization was calculated individually for the different oligonucleotides. Finally, the membrane was washed two times for 5 min with 3× SSC/0.1% SDS at room temperature and another two times for 30 min with 3× SSC/0.1% SDS at hybridization temperature −5°C. For exposure, the membrane was wrapped in Saran wrap and subjected to autoradiography or phosphoimaging.

RNA for Northern blot hybridization was separated on a denaturing 15% polyacrylamide gel before electrotransfer to a Hybond N+ membrane (Amersham Pharmacia Biotech). Transfer was carried out in a 0.25× TAE buffer (40 mM Tris-acetate 1 μM EDTA, pH 8) for 1 h at 500 mA. Membrane treatment, hybridization, and washes were performed as described for dot blots.

RNA Sequencing

For direct sequence determination 5 μg of total RNA from *M. rufus* and *M. robustus* were ethanol precipitated in the presence of 1 pmol of [5′-^{32}P]γ-ATP-labeled oligonucleotide Mr1, resuspended in 12 μl of TE buffer (10 mM Tris/HCl, pH 7.5, and 2 mM EDTA), and incubated at 95°C for 3 min and then at room temperature for 10 min. After addition of 3 μl of 10× primer extension buffer (500 mM Tris/HCl, pH 8.3, 80 mM $MgCl_2$, and 300 mM KCl) to a final volume of 15 μl, 3 μl of this mixture were added to 2 μl of the G-, A-, T-, C- and fill-in reaction mixtures, respectively (G-mix: 125 μM dNTPs

and 2 mM dideoxy (dd)GTP; A-mix: 125 μM dNTPs and 1 mM ddATP; T-mix: 125 μM dNTPs and 2 mM ddTTP; C-mix: 125 μM dNTPs and 500 μM ddCTP; fill-in-mix: 125 μM dNTPs). Finally, 0.25 μl of 200 mM dithiothreitol (DTT) and 3 U of AMV reverse transcriptase (Stratagene) were added to each reaction and incubated at 37°C for 1 h. The reactions were stopped with 4 μl of loading buffer (80% formamide, 10 mM Tris/HCl, pH 7.6, 0.25% xylenecyanol, and 0.25% bromphenol blue) and electrophoresed on a denaturing 10% polyacrylamide gel.

Sequencing of 3′-end–labeled tRNAs with base-specific nucleases was performed with the RNA Sequencing Enzyme Kit according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Purification of Mitochondria and Preparation of Mitoplasts

All steps were carried out at 4°C. Fresh liver material was minced with a scalpel blade and rinsed several times in 1× phosphate-buffered saline (137 mM NaCl, 27 mM KCl, 4.3 mM $Na_2HPO_4 \times 7H_2O$, and 1.4 mM KH_2PO_4) and two times in RSB (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, and 1.5 mM $CaCl_2$). Before tissue disruption, cells had 10 min to swell in RSB. Tissue disruption was achieved by four strokes (600 rpm) in a motor-driven glass/Teflon potter with 10 ml RSB/g tissue. After addition of an equal volume of MS (420 mM mannitol, 140 mM sucrose, 10 mM Tris/HCl, pH 7.5, and 5 mM EDTA, pH 7.5) nuclei and cell debris were precipitated three times by centrifugation at 1000 × *g* for 10 min. The supernatant was used to prepare mitochondria (Tapper *et al.*, 1983; Kiss and Filipowicz, 1992). Gradient-purified mitochondria were resuspended in M2 (20 mM HEPES, pH 7.7, 210 mM mannitol, 70 mM sucrose, 10 mM KCl, 1 mM DTT, 1.5 mM $MgCl_2$, and 3 mM $CaCl_2$) before measurement of the protein concentration (Bradford assay, Bio-Rad, Hercules, CA) with the use of bovine serum albumin as the standard.

For mitoplast preparation, mitochondria were incubated in the presence of digitonin, which selectively removes the outer membrane while leaving the inner membrane intact. In a titration assay, 0.2- 0.8 mg of digitonin (Fluka, Buchs, Switzerland) per mg of mitochondrial protein was added in the presence of 800 U/ml micrococcal nuclease (Amersham Pharmacia Biotech) to destroy the outer membrane and membrane-associated contaminating cytoplasmic RNA. The mixture was incubated for 30 min at room temperature with intermittent shaking. To inactivate micrococcal nuclease, EDTA and EGTA were added to a final concentration of 3 and 6 mM, respectively. Mitoplasts were collected by centrifugation at 10,000 × *g* for 10 min.

RNA dot blot hybridizations of the mitoplast preparations with a mitochondrial (12S rRNA) and a cytoplasmic oligonucleotide probe (vertebrate consensus sequence of cytoplasmic $tRNA^{Leu}$) indicated that the treatment of mitochondria with 0.2 mg digitonin/mg mitochondrial protein with micrococcal nuclease (800 U/ml) led to contamination-free mitoplast RNA. Therefore, RNA fractions for all of the following experiments were prepared from mitoplasts treated accordingly. In control incubations, mitochondria were incubated either with micrococcal nuclease only or with 1% Triton X-100 and micrococcal nuclease.

Preparation of Mitochondrial S100 Extracts

Purified mitochondria were resuspended in 1 M KCl, 20 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride and lysed by sonication with three bursts of a Branson cell disruptor for 30 s (output control 4, 30% pulse cycle). After centrifugation for 20 min at 20,000 × *g*, the lysate was dialyzed twice for 8 h against 2 l of 10% glycerol, 50 mM Tris/HCl, pH 8.0, 15 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT before centrifugation at 100,000 × *g* for 1 h. Protein concentration was determined by the Bradford assay (Bio-Rad).

In Vitro Aminoacylation

tRNA samples were deacylated by incubation for 5 min at 75°C in a buffer containing 10 mM Tris/HCl, pH 9.0, followed by ethanol precipitation. For aminoacylation with lysine, 15 μg of mitoplast tRNA were incubated for 20 min at 37°C with 60 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 5 mM ATP, 0.5 μg/μl bovine serum albumin, 40 μg of mitochondrial S100, and lysine in a 100-fold excess in a total volume of 20 μl. Aminoacylation of 15 μg of mitoplast tRNAs with isoleucine was performed as described by Degoul *et al*. (1998). Incubations were stopped on ice by the addition of NaCl to a final concentration of 500 mM, acetic acid to 100 mM, and 1% SDS. The mixture was extracted with acidic phenol (saturated in 100 mM sodium acetate, pH 4.5, 500 mM NaCl, and 1% SDS). All subsequent steps were performed under acidic conditions and low temperature to stabilize the tRNA-amino acid ester bond.

Oxidation of RNA

For oxidation of acylated and nonacylated 3′-ends with $NaIO_4$, RNA samples were dissolved at 0.5 μg/μl in oxidation buffer (10 mM NaAc, 10 mM $MgCl_2$, 1 mM EDTA, and 15 mM β-mercaptoethanol, pH 4.5) (Andrulis and Arfin, 1979). $NaIO_4$ suspended in the same buffer was added to the sample in a 1000-fold molar excess >3′-ends of tRNAs for quantitative oxidation. After incubation on ice for 20 min in the dark with intermittent shaking, the reaction was stopped by the addition of an equimolar amount of glucose. Nucleic acids were recovered by three subsequent ethanol precipitations, followed by extraction with acidic phenol. Finally, tRNAs were deacylated and radioactively labeled with T4 RNA ligase and [^{32}P]pCp (England and Uhlenbeck, 1978).

Circularization and Tagging of tRNAs

Circularization of total small RNAs was done as described previously (Yokobori and Pääbo, 1995a). Ligation of tRNAs to an *Eco*RI-linearized pUC 18 plasmid (tagging) and subsequent cDNA synthesis was performed as described by Hetzer and Mueller (1993).

RESULTS

The Lysyl-tRNA Gene in Marsupial Mitochondria

To investigate the structure of marsupial $tRNA^{Lys}$ genes, primers complementary to the flanking COII and ATP8 genes were designed and used to amplify the lysyl-tRNA genes from the North American opossum (*D. virginiana*) and 10 other marsupial species native to South America, Australia, Tasmania, and New Guinea. Sequence analyses of the amplification products revealed an average of 5.5 substitutions (12%) in the 3′-end of the COII gene and 5′-end of the ATP8 gene among marsupials and an average of 12 differences (26%) among other mammals (Table 1). Thus, the genes flanking the marsupial lysyl-tRNA gene are well conserved, considering that most substitutions concern third codon positions.

In contrast, the sequences of the marsupial $tRNA^{Lys}$ genes differ from those of other mammals in that they are only 53–59 bp in length (Figure 1A), whereas they are between 64 and 70 bp long in other mammals. Furthermore, a comparison of the $tRNA^{Lys}$ genes of the 11 marsupial species revealed that they differ by an average of 32%, indicating a higher variability than in neighboring protein genes (Table 1). This extensive variation is restricted to the $tRNA^{Lys}$ gene, because $tRNA^{Asp}$ genes differ by only 2.5% among seven marsupials.

Table 1. Substitution rates of three mitochondrial genes in marsupials, placentals, and monotremes

	Partial COII/ATP8 sequences (combined)		Mt. lysyl-tRNA		Mt. aspartyl-tRNA	
Animal species	11 marsupials	14 placentals, 1 monotreme	11 marsupials	14 placentals, 1 monotreme	7 marsupials	14 placentals, 1 monotreme
No. of nucleotides	46	45	46	58	57	57
Mpd in %	12	26	32	16	2.5	18

Mean pairwise sequence difference (mpd) for mitochondrial lysyl-tRNA and aspartyl-tRNA genes (only stem-forming nucleotides) and partial sequences of COII and ATP8 genes.

Inferred Secondary Structure of tRNA^{Lys} Transcripts

The mitochondrial tRNA for lysine is expected to carry the anticodon UUU to allow the recognition of the lysine codons AAA and AAG. However, when folded into a putative secondary structure, only three of the 11 marsupial tRNA sequences have the expected anticodon, whereas the others carry anticodons UCU, ACA, or AUA, which are unable to decode lysine codons (Figure 1). Alternative secondary structure folding does not result in tRNA-like structures with UUU anticodons. Furthermore, at the discriminator position 73 (numbering according to Sprinzl *et al.*, 1998), an adenosine, which is a major identity element of tRNA^{Lys} in *Escherichia coli* (McClain *et al.*, 1990), is found in only four of the 11 marsupial sequences. Thus, the anticodon UUU as well as the discriminator base A is missing in most of the marsupial species analyzed.

In addition, other unusual structural features are present in the inferred structures of the marsupial tRNAs^{Lys}. At positions 9, 33, and 37, which are conserved among particular mitochondrial tRNAs (Kumazawa and Nishida, 1993; Helm *et al.*, 2000), nonmarsupial lysyl-tRNAs carry adenosine residues at positions 9 and 37 and uracil at position 33 (Figure 1B, human tRNA^{Lys}). In contrast, among the 11 marsupial sequences, only 8 have an adenosine at position 9, 7 have a uracil at position 33, and 7 have an adenosine at position 37 (Figure 1). Furthermore, the anticodon loop in two of the sequences consists of eight instead of the conserved seven nucleotides and the DHU is replaced by loops of 3–11 bases in all marsupial species (Figure 1B).

These data indicate that the marsupial gene for the mitochondrial tRNA^{Lys} is evolving extremely rapidly compared with other marsupial tRNA genes as well as tRNA^{Lys} genes from other mammals. Apart from a structure corresponding to an acceptor stem, it fails to display structural features and identity elements conserved among mammalian tRNA^{Lys}.

Absence of Other Mitochondria-type tRNA^{Lys} Genes

In the complete mitochondrial genomes of two marsupials, no additional lysyl-tRNA gene is found (Janke *et al.*, 1994, 1997). To investigate whether a conventional mitochondrial tRNA^{Lys} might be encoded in the nuclear genome and potentially imported into mitochondria in marsupials, total DNA from *D. virginiana* was digested with the restriction enzymes *Bgl*II, *Eco*RI, and *Pvu*II, separated by electrophoresis, and transferred to a nitrocellulose membrane for Southern blot analysis. In a control experiment, mouse total DNA was tested. When an oligonucleotide complementary to the conserved region of the anticodon stem and loop of a conventional (nonmarsupial) mitochondrial tRNA^{Lys} was hybridized to the filter, bands appeared in the mouse DNA digests, whereas no signal was detectable in the opossum DNA digests (Figure 2A). Conversely, when an oligonucleotide directed against the mitochondrial tRNA^{Lys} gene from *D. virginiana* was hybridized to the filter, bands were detected in *D. virginiana* DNA but not in mouse DNA (Figure 2B). The size of the hybridization signals in both experiments corresponds to the restriction fragments of mitochondrial DNA containing the individual mitochondrial lysyl-tRNA gene. Thus, there is no evidence that alternative forms of the mitochondrial genome or nuclear genes in marsupials encode a lysyl-tRNA gene that carries a sequence typical for mitochondrial tRNA^{Lys} from nonmarsupial mammals.

The Mitochondrial tRNA^{Lys} Transcript in Marsupials

To investigate whether the transcript of the marsupial mitochondrial tRNA^{Lys} gene is recognized by tRNA-processing enzymes and released as a tRNA-like molecule, total cellular RNA from *D. virginiana* and mouse as well as *E. coli* tRNA was separated on a denaturing polyacrylamide gel and transferred to a nylon membrane. Two hybridizations were carried out with the same oligonucleotides used for Southern blot hybridizations. The oligonucleotide specific for the *D. virginiana* mitochondrial lysyl-tRNA gave a single signal with *D. virginiana* total RNA (Figure 3A) at a position corresponding to tRNA length. This indicates that the marsupial tRNA^{Lys} is released from the primary transcript. To monitor the presence of a conventional mtRNA^{Lys} molecule in marsupial cells, the filter was reprobed with an oligonucleotide complementary to the mitochondrial lysyl-tRNA from nonmarsupial mammals. In this case, a hybridization signal was observed in only the mouse RNA preparation, indicating that such a tRNA is not present in *D. virginiana* (Figure 3B).

Absence of RNA Editing

RNA-editing activities in mitochondria of protozoans and metazoans can generate functional tRNAs or even change the identity of one tRNA molecule toward another (Lonergan and Gray, 1993; Mörl *et al.*, 1995; Yokobori and Pääbo, 1995b; Börner *et al.*, 1996; Reichert *et al.*, 1998; Alfonzo *et al.*, 1999; Lavrov *et al.*, 2000). Thus, it is conceivable that the mitochondrial tRNA^{Lys} transcript in marsupials might be

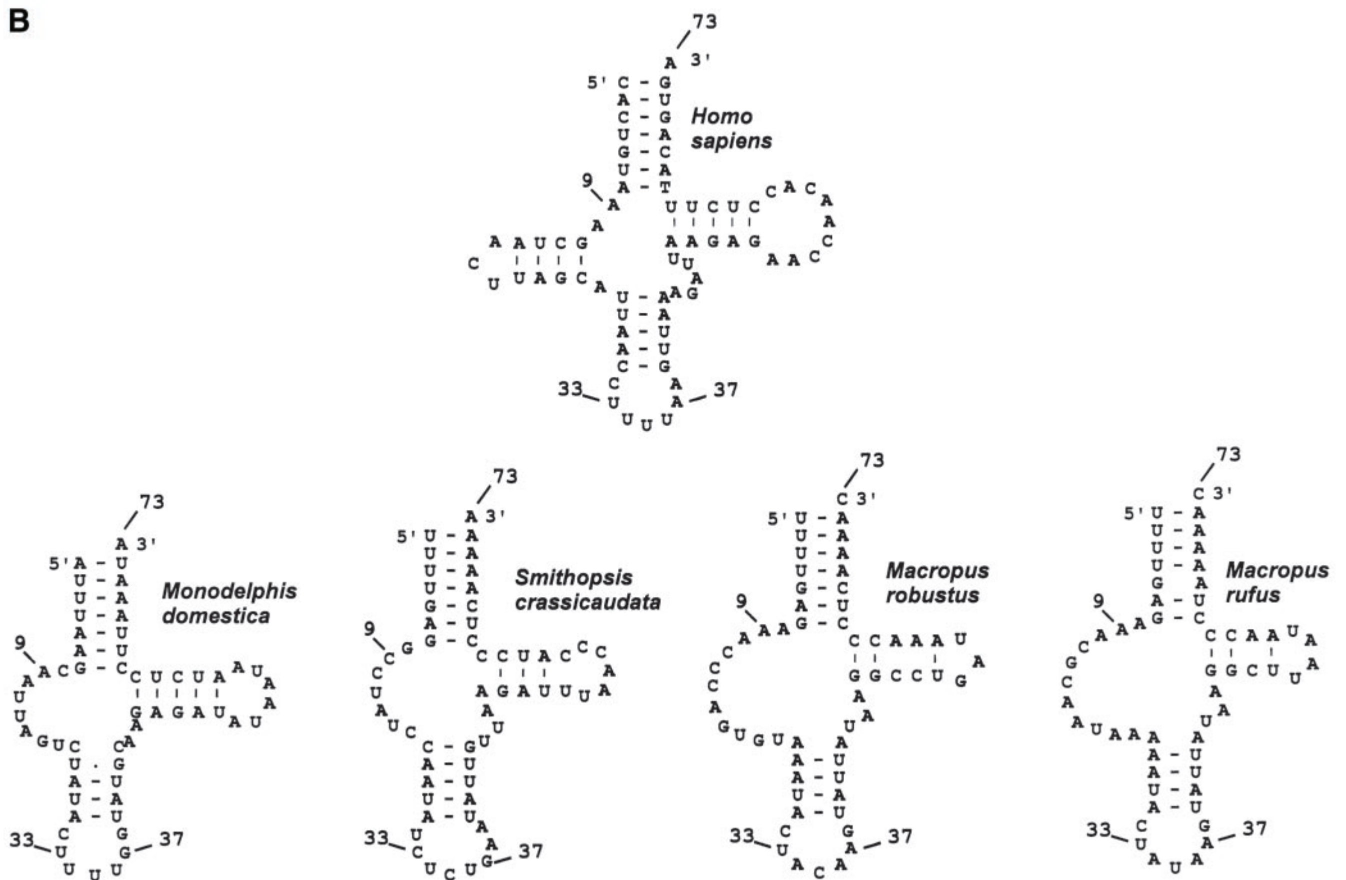

Figure 1. (A) Alignment of mitochondrial lysyl-tRNA genes from 11 marsupials. The *D. virginiana* sequence served as a reference. Dots indicate identity to the *D. virginiana* sequence, dashes indicate deletions, and substitutions are given by the individual nucleotide. The inferred anticodon is printed in red. Secondary tRNA structure elements are indicated above the alignment. (B) Inferred secondary structures of mitochondrial lysyl-tRNAs from *M. domestica* (anticodon UUU), *S. crassicaudata* (UCU), *M. robustus* (ACA), *M. rufus* (AUA), and human (UUU). Indicated positions 9, 33, 37, and 73 are described in the text. Numbering of the positions is according to Sprinzl *et al.* (1998) and does not correspond to the actual position in the tRNA. Note that the anticodon-loop of lysyl-tRNA from *S. crassicaudata* consists of eight instead of seven nucleotides.

converted into a functional tRNA by base changes or nucleotide incorporations. This possibility was assessed by three individual approaches, all involving the preparation of total cellular RNA, treatment with DNase, cDNA synthesis, PCR amplification, and sequencing.

For the first analysis, primers specific for the 3′-ends of mitochondrial $tRNA^{Lys}$ from *D. virginiana*, *P. tridactylus*, *M. rufus,* and *M. robustus* were used for cDNA synthesis. The cDNA was amplified by PCR (with the use of an oligonucleotide specific for the tRNA 5′-end as a second primer),

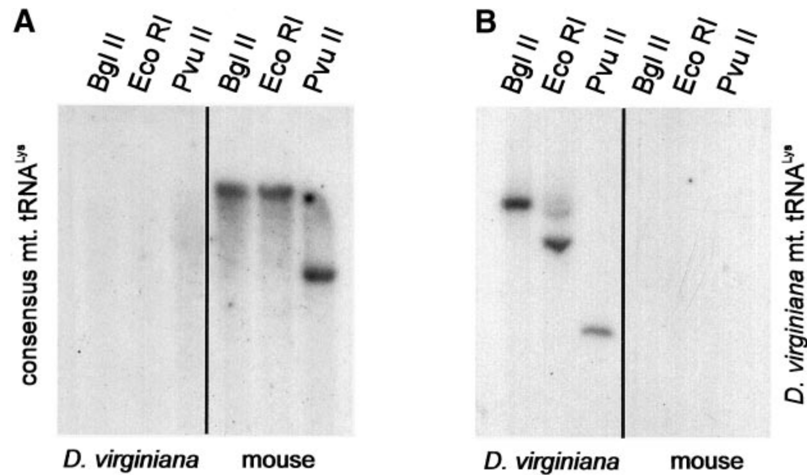

Figure 2. Southern blot hybridization of *Bgl*II-, *Eco*RI-, and *Pvu*II-digested total cellular DNA from *D. virginiana* and mouse. (A) Hybridization with radiolabeled oligonucleotide mt. K-cons. for the placental type of mitochondrial lysyl-tRNA. (B) Hybridization with oligonucleotide mt. K-op for *D. virginiana* mitochondrial lysyl-tRNA. Signals in A and B correspond to restriction fragments containing the individual mitochondrial gene.

and the resulting PCR product was subjected to sequence analysis. In a second approach, the transcripts from *M. robustus* and *M. rufus* were sequenced directly by reverse transcription with the use of only one primer specific for the tRNA 3′-end. The sequences obtained by both strategies were identical with the gene sequences and gave no indication of RNA editing (data not shown).

In a third approach, the cDNA sequence of the $tRNA^{Lys}$ acceptor stem from *M. domestica* was analyzed, thereby allowing the determination of the very 5′- and 3′-ends of the tRNA. This analysis was performed in two ways, one involving circularization of tRNAs and the other ligation of the tRNA 3′-end to an *Eco*RI-digested plasmid. The ligation products were amplified by reverse transcription/PCR and cloned. Sequence analysis of individual clones confirmed the genomic sequence of the $tRNA^{Lys}$ acceptor stem, giving no indication that this tRNA is converted to a conventional $tRNA^{Lys}$ by RNA editing. However, although 11 of 26 clones carried the tRNA-specific, posttranscriptionally added CCA terminus (or parts of it) at the 3′-end, the majority of clones (15/26) showed further 3′-terminal extensions of up to 11 apparently randomly assembled C and A residues (Figure 4). In a control experiment, the human mitochondrial $tRNA^{Lys}$ was analyzed in the same manner. In contrast to the *D. virginiana* tRNA, all cDNA clones carried a complete CCA-end (23 of 25) or parts of it (CC, 2/25), whereas none showed further 3′-extensions (Figure 4). Thus, the extended 3′-termini seem to be a feature of mitochondrial lysyl-tRNAs in marsupials and support the notion that these transcripts cannot be aminoacylated and do not represent functional tRNA molecules.

Because RNA editing in the anticodon can change the identity of a tRNA, other mitochondrial tRNAs carrying anticodons that could be changed to lysine (UUU) by base substitutions were analyzed in *D. virginiana*. Specifically, the tRNAs for glutamic acid (UUC) and glycine (UCC) could produce the UUU triplet by deamination of C residues in the anticodons. However, analysis of these molecules by reverse transcription/PCR and subsequent sequencing revealed no editing events in the anticodons (not shown). In addition, a similar analysis of tRNAs for asparagine (GUU) and glutamine (UUG), in which the replacement of one G by U in the anticodon could generate a lysyl-anticodon, showed no evidence of RNA editing (not shown).

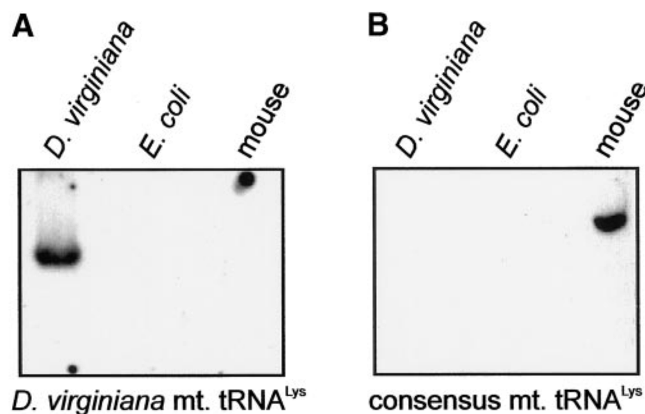

Figure 3. Northern blot hybridization of total cellular RNA from *D. virginiana* and mouse. *E. coli* tRNAs are included to serve as hybridization background controls. (A) Hybridization with oligonucleotide mt. K-op for *D. virginiana* mitochondrial lysyl-tRNA. (B) Hybridization with radiolabeled oligonucleotide mt. K-cons. for the placental type of mitochondrial lysyl-tRNA.

A tRNA for Lysine in Marsupial Mitoplasts

Because the single mitochondria-encoded $tRNA^{Lys}$ gene does not generate a functional lysine-accepting tRNA, the question remains which tRNA serves as $tRNA^{Lys}$ in marsupial mitochondria. To resolve this, an experimental approach was developed that identifies the tRNA and its sequence by the ability to accept lysine in an in vitro aminoacylation assay (Figure 5). The method takes advantage of the fact that nonprotected 3′-OH ends of RNA can be oxidized quantitatively by sodium periodate.

To avoid contamination by cytoplasmic RNA molecules, a purification procedure for mitoplasts that involves treatment with digitonin and micrococcal nuclease after enrichment of mitochondria by differential centrifugation and density gradient ultracentrifugation was used.

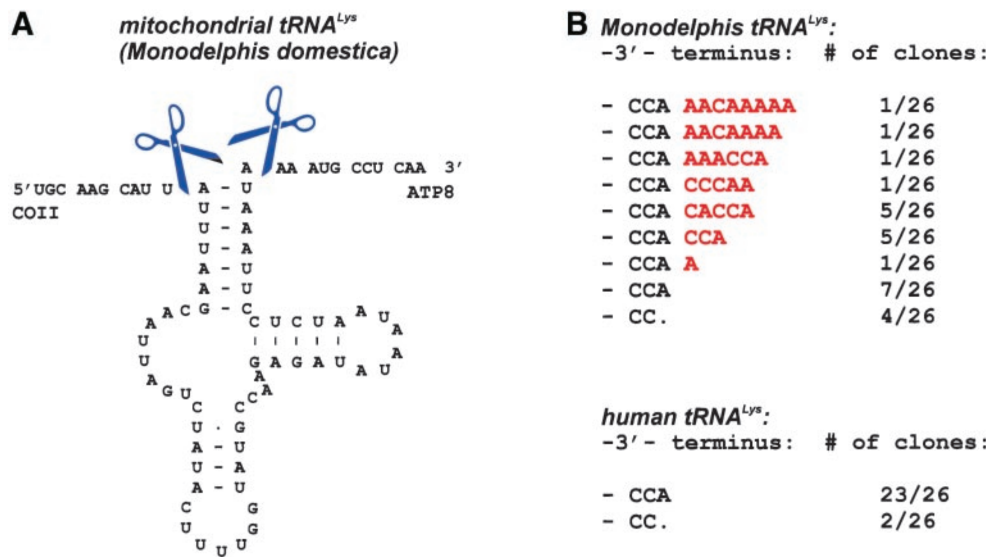

Figure 4. (A) Secondary structure drawing of mitochondrial tRNA^{Lys} from *M. domestica* within the precursor transcript. Scissors indicate 5′- and 3′-processing cleavage positions as determined from cDNA clone analysis after circularization or tagging of the tRNA. (B) Posttranscriptional addition of the CCA terminus and further 3′-terminal extensions (shown in red). Numbers indicate the frequency at which individual clones were observed.

To identify the tRNA with lysyl-identity in marsupial mitochondria, a deacylated mitoplast tRNA fraction from *M. domestica* was aminoacylated with lysine in vitro by a mitochondrial S100 extract of the same species. Thereby the corresponding tRNAs become protected at the 3′-OH end by esterification with the amino acid, and the remainder of tRNA molecules, possessing free 3′-OH ends, can be oxidized by sodium periodate. After subsequent deacylation only tRNAs that were esterified with lysine harbor a 3′-OH end available for labeling with [^{32}P]pCp (Figure 5). As controls, an aliquot of deacylated starting tRNAs was labeled radioactively without being aminoacylated and oxidized, and one deacylated tRNA aliquot was oxidized but not aminoacylated before labeling. Subsequently, all labeled fractions were separated on a denaturing polyacrylamide gel and visualized by autoradiography (Figure 6A). The single band was visible in the sample aminoacylated with lysine. This product was sequenced with the use of nucleotide-specific ribonucleases. Although the anticodon sequence was not readable (probably due to modifications), the sequence was highly similar to the human cytoplasmic tRNA$_{TTT}$ for lysine (Figure 6B). In a control experiment in which isoleucine was used in the aminoacylation reaction, the labeled band was unambiguously identified as the mitochondrial tRNA for isoleucine (not shown).

DISCUSSION

According to the complete mitochondrial sequence of *D. virginiana* (Janke *et al.*, 1994), 94 lysine codons exist in protein-coding genes, representing a codon frequency of 2.5%. This frequency is similar to lysine codon occurrence in human (2.5%), mouse (2.7%), cow (2%), and seal (2.7%) mitochondria (Wolstenholme, 1992). Furthermore, the positions of these codons are to a major extent consistent with the presence of conserved lysine codons in other vertebrate mitochondrial genomes, indicating that a functional tRNA^{Lys} is critical for mitochondrial function in marsupials. However, the gene that according to its location in the

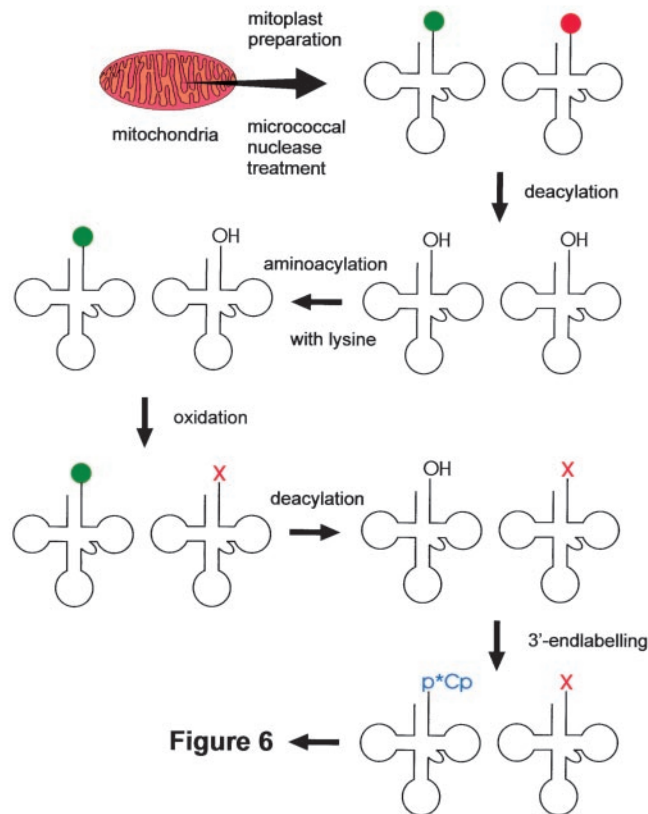

Figure 5. Strategy to identify the nature of the mitochondrial lysyl-tRNA. tRNAs were isolated from highly purified mitoplasts from *M. domestica* and deacylated (green dots indicate lysine; red dots denote any amino acid except lysine). tRNAs were charged with lysine (green dot) on incubation with a mitochondrial S100 extract. The 3′-OH groups of nonaminoacylated tRNAs were blocked by oxidation with the use of $NaIO_4$ (red X). Subsequently, the tRNA protected by lysine was deacylated, leading to a free 3′-OH group that can be selectively labeled by pCp ligation.

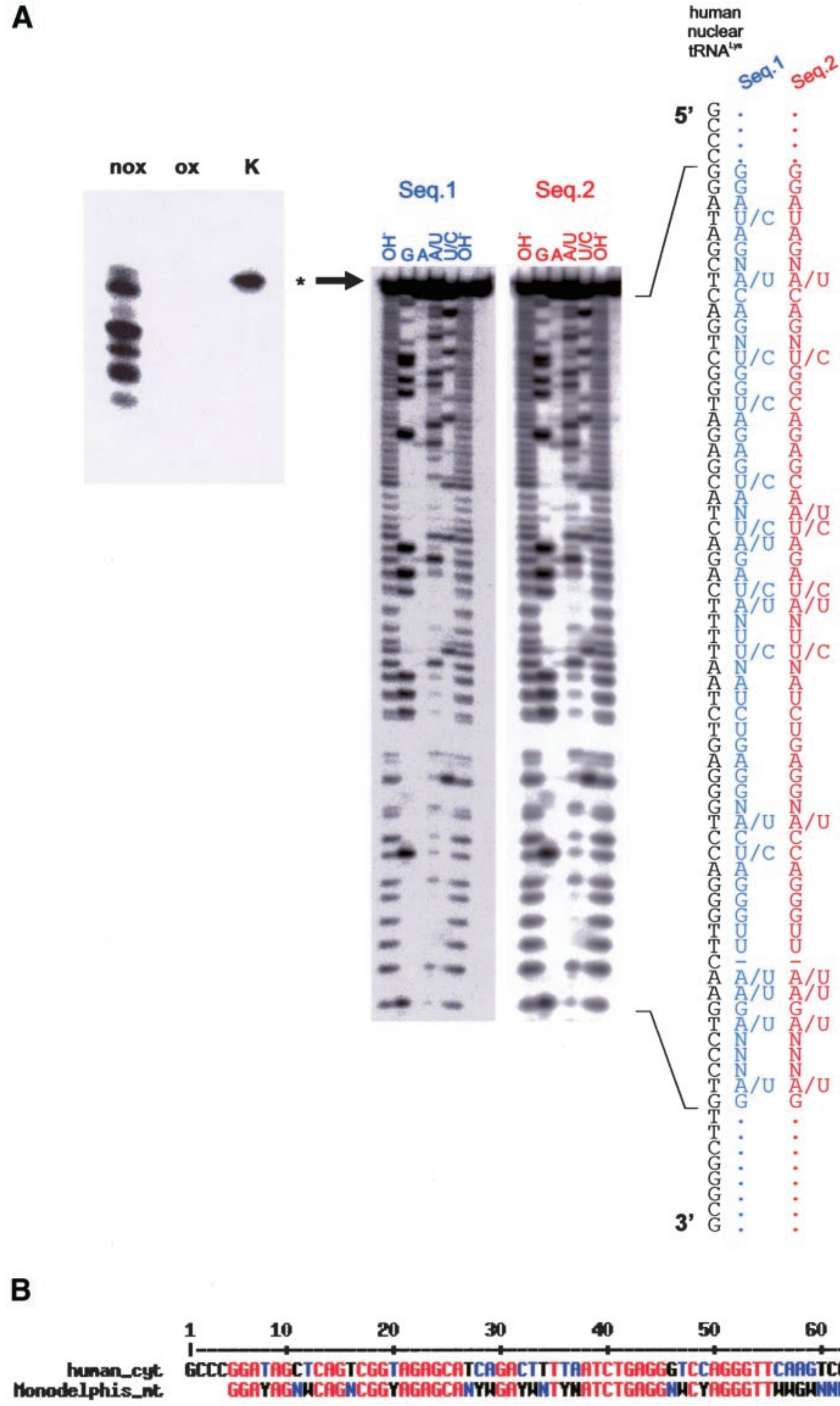

Figure 6. (A) In vitro aminoacylation of deacylated mitochondrial tRNA fractions from *M. domestica* with lysine, followed by periodate oxidation of nonprotected molecules. Lane nox designates mitochondrial tRNAs that were labeled without prior aminoacylation or oxidation. Lane ox indicates that mitochondrial tRNAs were oxidized quantitatively with a 1000-fold molar excess of $NaIO_4$ so that no molecules could be labeled at the 3′-end. Mitochondrial tRNA fraction of lane K was aminoacylated in vitro with lysine before oxidation. Aminoacylated, oxidation-resistant tRNAs were deacylated and 3′-end labeled. The band indicated by the asterisk was isolated for sequence analysis (two reactions) and identified as a nuclear type of $tRNA^{Lys}_{(TTT)}$. (B) Alignment of the human nuclear $tRNA^{Lys}$ and the obtained sequence of the marsupial $tRNA^{Lys}$ associated with mitochondria. The alignment was done according to Corpet (1988). Human cyt, human cytosolic $tRNA^{Lys}$; Monodelphis mt, mitochondria-associated $tRNA^{Lys}$ in *Monodelphis*; Y = C or U; W = A or U.

mitochondrial genomes corresponds to the lysyl-tRNA gene in 11 marsupials is highly variable in sequence compared with lysyl-tRNA genes in placentals and monotremes. Thus, although the neighboring protein-coding genes (COII and ATP8) as well as other tRNA genes in marsupials evolve at a rate similar to that in placentals and monotremes, the gene for $tRNA^{Lys}$ evolves much faster (Table 1). Additionally, the marsupial version of this gene has a reduced size in comparison to mammalian copies, primarily due to the lack of a DHU arm in the inferred secondary structure. tRNAs lack-

ing the DHU arm are well known in vertebrate mitochondria (tRNA^{Ser}[AGY]; Helm *et al.*, 2000) as well as in nematode mitochondria (all tRNAs; Dirheimer and Martin, 1990). They can adopt a higher order tRNA structure that is functional in translation (Watanabe *et al.*, 1994). Therefore, a missing DHU stem in marsupial mitochondrial tRNA^{Lys} per se does not implicate a nonfunctional molecule. However, the inferred secondary structures as well as the alignment of the marsupial lysyl-tRNA gene sequences (Figure 1) revealed that only three of 11 possess the lysyl-tRNA anticodon UUU, whereas the others have anticodons UCU, ACA, and AUA, which cannot recognize lysine codons (Jukes, 1995). Additionally, none of several other conserved nucleotides is present consistently in the marsupial tRNA^{Lys}, whereas they are conserved in placental and monotreme lysyl-tRNAs. In effect, the only structural part of the marsupial tRNA^{Lys} genes that is conserved is the acceptor stem, which is composed of 6–7 bp. The conservation of this part of the molecule might indicate the necessity for tRNA-processing reactions at the RNA precursor level to release the neighboring COII and ATP8 transcripts. In conclusion, the genes and inferred products of mitochondrial lysyl-tRNA in marsupials display many unusual features that are not consistent with a functional lysyl-tRNA. The DNA sequence probably represents a mitochondrial pseudogene.

Because an additional gene for a conventional mitochondrial lysyl-tRNA could be detected neither in the complete mitochondrial genome nor in the nuclear DNA of *D. virginiana*, the only remaining possibilities to provide a functional tRNA^{Lys} in marsupial mitochondria are either identity switch of a nonlysyl-tRNA by editing or the import of a nuclear-encoded tRNA^{Lys}. Because in marsupial mitochondria the aspartyl-tRNA identity is generated by a C to U conversion at the second position of the anticodon of a tRNA^{Gly} (Mörl *et al.*, 1995; Börner *et al.*, 1996), a similar situation is conceivable also for the mitochondrial lysyl-tRNA. However, several experimental approaches failed to detect any editing reaction in the mitochondrial tRNAs for glutamic acid (UUC) or glycine (UCC), where one or two deamination reactions would restore a UUU anticodon. In addition, sequence analysis of the anticodons of the tRNAs for asparagine (GUU) and glutamine (UUG), in which a single G > U change would lead to the missing lysyl-anticodon, revealed no evidence for any RNA editing events. Therefore, an editing mechanism that creates a functional tRNA^{Lys} by changing the identity of another mitochondrial tRNA seems to be excluded. Furthermore, the conversion of the tRNA^{Lys} pseudogene transcript into a functional lysyl-tRNA by a massive editing is also highly unlikely, because cDNA clones of this transcript showed no deviation from the genomic sequence and Northern blots failed to detect any tRNA carrying a conserved mitochondrial tRNA^{Lys} sequence. Furthermore, the 3′-ends of the majority of the tRNA^{Lys} transcripts carried (in addition to the common CCA terminus) up to 11 additional A and C residues in various orders, some of them resembling short polyA tails. In *E. coli*, polyadenylation is used as a signal for RNA degradation (O'Hara *et al.*, 1995; Ingle and Kushner, 1996). Because the pseudo-tRNA^{Lys} transcripts are probably not aminoacylated, it is likely that they represent nonfunctional molecules and it is tempting to speculate that the elongated forms represent molecules that are tagged for degradation, a situation that would imply the existence of an RNA decay mechanism in marsupial mitochondria similar to *E. coli*.

The remaining possibility that can deliver a functional tRNA^{Lys} to marsupial mitochondria is the import of a nuclear-encoded version of this tRNA. The fact that a single tRNA version associated with highly purified mitochondria, which is chargeable with lysine in vitro is a nuclear-encoded tRNA for lysine, represents evidence for tRNA import into marsupial mitochondria.

Mitochondrial tRNA import exists in yeast, protozoa, and plants (Hancock and Hajduk, 1990; Dietrich *et al.*, 1992; Tarassov and Entelis, 1992). It can comprise different numbers of tRNAs, from one single tRNA to a complete tRNA set (Schneider and Maréchal-Drouard, 2000). In mammalian mitochondria, the RNA subunit of RNase P and RNase MRP as well as the 5S rRNA is imported (Doersen *et al.*, 1985; Li *et al.*, 1994; Magalhaes *et al.*, 1998). It is furthermore noteworthy that in yeast a cytosolic tRNA^{Lys} is imported into mitochondria, although its function in the organelle remains unclear, because, first, the yeast mitochondrial genome contains a full set of tRNA genes and, second, the yeast mitochondrial lysyl-tRNA synthetase does not recognize, and therefore cannot aminoacylate, this tRNA (Tarassov *et al.*, 1995; Tarassov and Martin, 1996).

In the case of the marsupial mitochondria, the imported cytosolic tRNA^{Lys} must be recognized by the mitochondrial lysyl-tRNA synthetase so as to compensate for the missing mitochondrial tRNA gene. In humans, a single gene encodes both the cytoplasmic and the mitochondrial lysyl-tRNA synthetases (Tolkunova *et al.*, 2000). Thus, a single synthetase aminoacylates both the cytosolic and the mitochondrial version of the corresponding lysyl-tRNA. It is likely that a similar situation exists in marsupials: the import of both the tRNA^{Lys} and the corresponding tRNA synthetase would be reminiscent of the situation in yeast, in which a complex consisting of the aminoacylated tRNA^{Lys} and the precursor of the mitochondrial lysyl-tRNA synthetase is imported (Tarassov *et al.*, 1995; Tarassov and Martin, 1996). However, although this synthetase is needed for the import of the yeast tRNA^{Lys}, it seems that it cannot charge the tRNA. Rather, it is used as a carrier for tRNA^{Lys} import into yeast mitochondria. Furthermore, it could not be shown that the imported cytoplasmic tRNA is functional on yeast mitochondrial ribosomes, whereas the mitochondria-encoded tRNA^{Lys} seems to be functional (Martin *et al.*, 1979; Kolesnikova *et al.*, 2000). Therefore, the function of the imported tRNA^{Lys} in yeast mitochondria is currently unclear, whereas in marsupials, it seems to be a prerequisite for mitochondrial translation.

Taken together, our results suggest that mitochondrial import of cytosolic tRNAs is not restricted to yeast, protozoans, and plants but occurs also in marsupials. Further experiments will clarify the molecular mechanisms involved in the import process and elucidate whether it occurs also in other mammalian groups. Such insight might help to develop a system to introduce tRNAs and maybe other nucleic acids into mammalian mitochondria.

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