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RESEARCH ARTICLE

Characterization and localization of mitochondrial DNA-encoded tRNAs and nuclear DNA-encoded tRNAs in the sea anemone *Metridium senile*

C. Timothy Beagley · David R. Wolstenholme

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Abstract The mitochondrial (mt) genome of the sea anemone *Metridium senile* contains genes for only two transfer RNAs (tRNAs), tRNAf-Met and tRNATrp. Experiments were conducted to seek evidence for the occurrence of functional tRNAs corresponding to these genes and for the participation of nuclear DNA-encoded tRNAs in mt-protein synthesis. RNA sequences corresponding to the two mt-tRNA genes were located in mitochondria and it was shown that 3′-CC (and possibly A, but no other nucleotide) is added post-transcriptionally to the 3′ end of at least 50 % of mt-tRNAf-Met molecules and to a small fraction of the mt-tRNATrp molecules. Using specific oligonucleotide primers based on expected nuclear DNA-encoded tRNAs in a series of RACE experiments, we located the nuclear genes for tRNAGln, tRNAIle, tRNAi-Met, tRNAVal and tRNAThr. Data from Northern blot analyses indicated that mtDNA-encoded tRNAf-Met is limited to mitochondria but that nuclear DNA-encoded tRNAVal and tRNAi-Met are present in the cytoplasm and in mitochondria. These data provide direct evidence that in *M. senile*, mature, functional tRNAs are transcribed from the mtDNA-encoded tRNAf-Met and tRNATrp genes, and are consistent with the interpretation that both nuclear DNA-encoded tRNAVal and tRNAi-Met are utilized in mitochondrial and cytosolic protein synthesis.

Keywords Mitochondrial tRNAs · Nuclear tRNAs · tRNA genes · CCA addition · Importation

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Introduction

The mitochondrial (mt) genomes of almost all bilaterian animals comprise a single circular molecule of 14–24 kb that contains the genes for 13 energy pathway proteins and the two RNA components of the mt-ribosome, as well as 22 transfer RNAs (tRNAs) that appear to be sufficient to decode the mt-protein genes (for references, see Barrell et al. 1979, 1980; Anderson et al. 1981; Wolstenholme 1992; Wolstenholme and Fauron 1995; Boore 1999; Gissi et al. 2008; Bernt et al. 2012). A similar mt-genome in regard to size, conformation, and protein gene and rRNA gene content was found to occur in the sea anemone *Metridium senile* (subclass Hexacorallia, Anthozoa, Cnidaria) (Beagley et al. 1996, 1998). However, a notable difference was that this genome encodes only two transfer RNAs (tRNAf-Met and tRNATrp). This has since been shown to be a common feature of the mt-genomes of hexacorallian mtDNAs, and that mt-genomes of the anthozoan subclass Octocorallia encode only tRNAf-Met (Beagley et al. 1995; Beaton et al. 1998; Lavrov 2007). Although among bilateria, such a large deficiency in mt-tRNA genes has only been recorded for a chaetognathan (Helfenbein et al. 2004), mt-genomes lacking various numbers of tRNA genes have been recorded for a number of organisms including the protozoans, *Tetrahymena pyriformis*, *Leishmania tarentolae*, and *Trypanosoma brucei*, some sponges and angiosperm plants, and in some cases evidence for importation of nuclear DNA-encoded tRNAs into mitochondria has been obtained (Marechal-Drouard et al. 1988, 1990; Simpson et al. 1989; Hancock and Hajduk 1990; Burger et al. 1995; Tarassov et al. 2007; Lavrov 2007, 2011; Adshya 2008; Alfonzo and Soll 2009; Duchene et al. 2009). However, experimental data regarding the involvement of mtDNA-encoded tRNAs and nuclear

DNA-encoded tRNAs in cnidarian mt-protein synthesis is lacking. Therefore, we undertook the presently reported study to elucidate the likely involvement of tRNAs transcribed from the two mt-tRNA genes and from nuclear tRNA genes in *M. senile* mt-protein synthesis.

In addition to a reduction in tRNA genes, the mt-genomes of bilateria exhibit a number of unusual features that include extreme compaction of the component genes, genetic codes that have up to five changes in codon specificity relative to the standard (universal) genetic code, unorthodox translation initiation codons, and replication by a unique asymmetrical mode (for references, see Wolstenholme 1992; Beagley et al. 1998; Brown et al. 2005; Watanabe and Yokobori 2011; Bernt et al. 2012; Abascal et al. 2012). A further, notable general feature of bilaterian mt-genomes is the considerable diversity of structure of the encoded tRNAs relative to standard tRNAs (i.e., including tRNAs encoded in DNAs of prokaryotes, eukaryotic nuclei, plant chloroplasts and plant mitochondria; Sprinzl et al. 2005; Juhling et al. 2009). Most bilaterian mt-tRNAs have a reduced dihydrouridine loop that lacks the usually conserved G_{18}–G_{19} pair and a TψC loop in which the usually conserved 5′-$G_{53}T_{54}T_{55}C_{56}R_{57}A_{58}N_{59}Y_{60}C_{61}$ sequence is either reduced or totally lacking (Wolstenholme 1992; Juhling et al. 2009). In all bilaterian mt-tRNAs that recognize AGN (Serine) codons, and occasionally mt-tRNAs that recognize UCN (serine) codons, the dihydrouridine arm is replaced by a simple loop of between 2 and 13 nucleotides (De Bruijn et al. 1980; Wolstenholme 1992; Beagley et al. 1999). A more extreme situation is that in all tRNAs encoded in mtDNAs of the nematode worms *Ascaris suum* and *Caenorhabditis elegans* and other closely related nematodes, with the exception of those tRNAs recognizing serine-specifying codons, the TψC arm is replaced with a simple loop of between 6 and 12 nucleotides (Wolstenholme et al. 1987, 1994; Okimoto et al. 1990; Montiel et al. 2006). Further, sequences in mtDNA molecules of species of enoplean nematodes have been interpreted as encoding some tRNAs in which the dihydrouridine and TψC arm are each replaced with a simple loop of nucleotides (Juhling et al. 2009). Interestingly, primary and predicted secondary structures of the tRNAs encoded in cnidarian mtDNAs more closely resemble standard tRNAs rather than any of the sorts of reduced mt-tRNAs found in bilateria (Pont-Kingdon et al. 1994, 1998, 2000; Beagley et al. 1995, 1998).

All mature tRNAs examined, regardless of their genetic origin, posses a 3′-terminal CCA which is required for amino acid addition (Tamura et al. 1994; Liu and Horowitz 1994; Nagaike et al. 2001), for base pairing interaction of the tRNA with the ribosome (Tamura 1994; Samaha et al. 1995), and for deacetylated tRNAs to leave the ribosome (Lill et al. 1988). In many prokaryotes and some archaea, the 3′-terminal CCA is encoded in all or some tRNA genes (Marck and Grosjean 2002). In contrast, neither mt-tRNA genes nor nuclear DNA-encoded tRNA genes of metazoa have CCA at their 3′ ends; rather CCA is added post-transcriptionally by nucleotidyl transferases (Deutscher 1990; Martin 1995; Tomita and Weiner 2001).

Although the mt-genome of *M. senile* contains the same circularly arranged, compact gene complement as that of most bilateria, besides the deficiency of tRNA genes, this genome was found to have a further unusual feature (Beagley et al. 1996, 1998). Each of two protein genes, *cox1* and *nad5* contain a group 1 intron. The *cox1* intron encodes a putative homing endonuclease, and the *nad5* intron contains the genome's only copy of two other protein genes, *nad1* and *nad3*. An *nad5* intron alone, or both a *cox1 intron* and an *nad5* intron have since been located in other hexacorallian mtDNAs but not in the mtDNAs of other Cnidaria (Beagley et al. 1996; Lavrov 2007). Also, with the exception of rare TGA codons that appear to specify tryptophan (as in all other metazoan mt-genetic codes), the *M. senile* mt-protein genes utilize a standard genetic code.

That there is, in fact, a transcription–translation system in *M. senile* mitochondria that results in the generation of functional mtDNA-encoded proteins is fully supported by the processing of primary transcripts to eliminate introns and provide complete and uninterrupted transcripts of the *nad5*, *nad1* and *nad3* genes (Beagley et al. 1996), by uninterrupted open reading frames in all other mt-protein genes, and by the occurrence of genes for rRNAs that have the potential to fold into secondary structures consistent with other prokaryotic and metazoan mt-rRNAs (Pont-Kingdon et al. 1994; Beagley et al. 1996, 1998). Further support for this contention has been provided by bioinformatic evidence indicating the occurrence in a hexacoralian cnidarian of a nuclear DNA-encoded aminoacyl-tRNA synthetase specific to the mtDNA-encoded tRNATrp and nuclear DNA-encoded aminoacyl-tRNA synthetases that include mitochondrion-targeting sequences (Haen et al. 2010).

Because the *M. senile* mt-genome contains only two tRNA genes, we considered it worthwhile to seek direct evidence as to whether one or both are, in fact, transcribed and processed into tRNAs that could function in protein synthesis. We provide experimental data indicating that in *M. senile* mitochondria, there occur tRNAs corresponding in sequence to the mt-tRNAf-Met and mt-tRNATrp genes that at their 3′ ends have post-transcriptionally added nucleotides expected for them to be functional in protein synthesis. Further, we provide evidence that mt-tRNAf-Met is confined to mitochondria but that nuclear-encoded tRNAVal and tRNAi-Met are located in both the cytoplasm and mitochondria indicating them to be needed for both cytoplasmic and mt-protein synthesis.

Materials and methods

Preparation of RNA fractions

Preparation of mitochondria from *M. senile* and isolation of RNA from mitochondria (mt-RNA) followed previously described (Pont-Kingdon et al. 1994). Isolation of whole cell RNA (wc-RNA) was achieved using the REX™ total RNA extraction kit (Amersham Life Sciences). RNA was also isolated from a cytoplasmic fraction from which mitochondria had been removed as follows. A single (3 cm diameter) *M. senile* was homogenized in a Waring blender at high speed for 5 s in ice cold 10 % sucrose TE. The homogenate was immediately centrifuged for 10 min at 10,000*g* to sediment any undisrupted cells, and mitochondria. The supernatant was extracted three times with phenol–chloroform–isoamyl alcohol (PCI; 50:50:1) and the final aqueous phase was precipitated with 2.5 volumes of 95 % ethanol.

RNA preparations were incubated in 0.5 M Tris, pH 8.7 for 30 min at 37 °C to discharge amino acids from tRNAs (Norma Wells, personal communication). The RNA preparations were then extracted once with PCI and the aqueous phases were passed through a Centricon 100 microconcentrator (Amicon). RNA molecules in the flow-through were concentrated with a Centricon 30 microconcentrator and distribution of sizes of molecules in each retentate was determined by electrophoresis through a denaturing (15 % polyacrylamide, 8 M urea) gel (Fig. 1). RNA molecules in the microconcentrator-selected fraction ranged in size from approximately 40 to 160 nt, and the predominant size classes are similar to those of *Escherichia coli* tRNAs (Fig. 1).

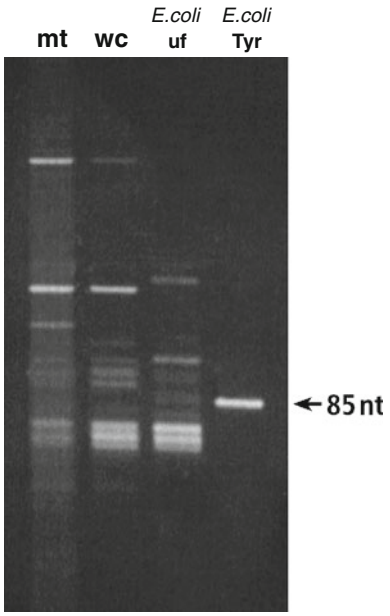

Fig. 1 Size profiles of RNA molecules isolated from a mitochondrial (*mt*) fraction and from whole cells (*wc*) of *Metridium senile*. An ethidium bromide-stained denaturing (15 % polyacrylamide, 8 M urea) gel following electrophoresis of RNA molecules is shown. *Lanes mt* and *wc* contain 1 µg each of RNA molecules isolated from a mt-fraction and from whole cells, respectively, which in both cases were selected by passage through a Centricon 100 microconcentrator (Amicon) and concentrated with a Centricon 30 microconcentrator. *Lane E. coli uf* contains 1 µg of unfractionated *Escherichia coli* tRNAs (Sigma) and *Lane E. coli Tyr* contains 100 ng of *E. coli* tRNATyr (Sigma) with an expected size of *85 nt* (Sprinzl et al. 2005)

Determination of mt-tRNA 3′-end sequences

3′ Rapid amplification of cDNA ends (RACE); (Frohman 1990) analyses were carried out to determine the 3′ ends of *M. senile* mtDNA-encoded tRNAf-Met and tRNATrp, following the procedures described previously (Pont-Kingdon et al. 1994; Beagley et al. 1996, 1998). RNA molecules in the 40–160 nt mt-RNA preparation were polyadenylated in a 30-µl reaction containing 1 µg RNA, 20 U RNasin (Promega), 0.25 mM ATP, 1,500 U cloned yeast Poly-A polymerase (Amersham Life Sciences) and the suppliers reaction buffer, at 30 °C for 30 min. Following a second PCI extraction, the polyadenylated RNA was desalted and concentrated with a Centricon 30 microconcentrator, then reverse transcribed in a 25-µl reaction containing 20 U AMV reverse transcriptase (Amersham Life Sciences), 20 U RNasin, 0.5 mM each of dATP, dGTP, dCTP and dTTP and 0.5 pmol primer TB17-1 [an oligonucleotide containing *Bam*HI, *Bgl*II and *Pst*I restriction sites (underlined):

5′-CCAGATCTGGATCCTGCAGTTTTTTTTTTTTTT TT (Beagley et al. 1996)] in the supplied reverse transcriptase reaction buffer for 5 min at 25 °C, and then for 10 min at 45 °C. The reaction mixture was then extracted with PCI, concentrated with a Centricon 30, and the retentate (designated the 3′-RACE pool) was brought to 100 µl with TE and stored at 4 °C.

The 3′-end region of each mt-tRNA sequence was amplified by polymerase chain reaction (PCR) in a 100-µl reaction containing 2 µl of the 3′-RACE pool, 0.2 mM each of dATP, dGTP, dCTP and dTTP, 2.5 U AmpliTaq™ DNA polymerase (Perkin Elmer) and 25 pmol each of a primer comprising the *Bam*HI, *Bgl*II and *Pst*I site-containing segment of TB17-1 (TB17-2: 5′-CCAGATCT GGATCCTGCAG) and a sense oligonucleotide primer: for the mt-tRNAf-Met, TMB-2 [5′-TGGGAATTCACC-AGA CTCATGATCT (see Fig. 2)] which contains two mismatched nucleotides (underlined) to create an *Eco*RI restriction site is used for subsequent cloning of the double-stranded PCR product; for tRNATrp, TBW-2 (5′-AAGG

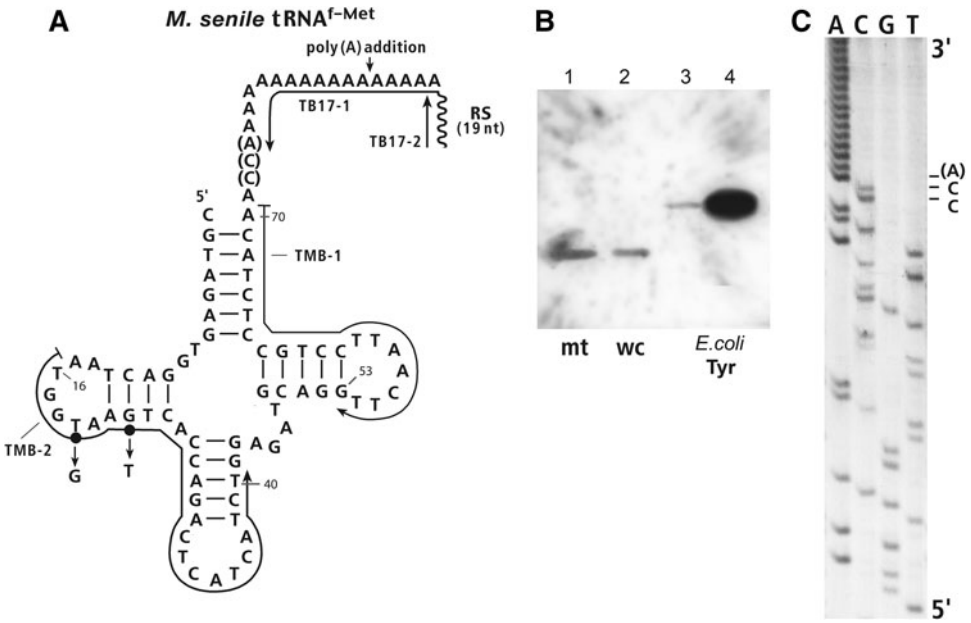

Fig. 2 Northern blot and 3′-RACE analysis of *M. senile* mt-tRNAf-Met. **a** The *M. senile* mt-tRNAf-Met gene shown in the presumed secondary structural form of the corresponding tRNA (Pont-Kingdon et al. 1994; Beagley et al. 1998) to which the three nucleotides CCA (each in *parenthesis*) sequentially at the 3′ end and 16 As representing polyadenylation by yeast poly-A polymerase are added. The two *solid lines* TMB-1 and TMB-2 on the outside of the sequence identify segments to which oligonucleotide primers were made, with the *arrowhead* indicating the 5′–3′ direction. The *solid line* TB17-1 is a poly-A primer with a 3′-*Eco*RI restriction site (RS)-containing 19 nt sequence. The *short solid line* TB17-2 is a primer complementary to the 3′ *Eco*RI site-containing sequence of TB17-1. The *solid dots* in primer TMB-2 indicate changes to the tRNAf-Met gene sequence made to create an *Eco*RI site. **b** Northern blot hybridizations. *Lanes 1* and *2* contain RNA isolated from a mitochondrial fraction (*mt*), and whole, unfractionated cells (*wc*), and *lanes 3* and *4* contain purified *E. coli* tRNATyr, electrophoresed through a denaturing (15 % polyacrylamide, 8 M urea) gel. *Lanes 1* and *2* were probed with ^{32}P-labeled oligonucleotide TMB-1 and *lanes 3* and *4* were probed with a ^{32}P-labeled oligonucleotide that is complementary to nucleotides 46–66 or *E. coli* tRNATyr (Sprinzl et al. 2005). **c** Autoradiograph showing the product of sequencing a clone of a cDNA of the 3′-end proximal region of mt-tRNAf-Met that includes the post-transcriptionally added CC (and presumably A), shown to the *right* of the sequence, and the synthetically added poly(A) tail

AAGATCTTTAGCCTTCAAAGC) and TBW-3 (5′-AGC TAAAACTGCAGGTTCAAGT), each containing two mismatched nucleotides (underlined) to create a *Bgl*II restriction site and a *Pst*I restriction site, respectively. Cycling was 45 s at 94 °C, 45 s at 60 °C, and 45 s at 72 °C. The double-stranded PCR products were sequenced by the dideoxynucleotide chain-terminating method (Sanger et al. 1977) using Sequenase™ (Amersham Life Sciences).

RACE amplification of nuclear DNA-encoded tRNA sequences

Fractions of small (40–160 nt) wc-RNAs were 3′ polyadenylated, and oligo-dT primed cDNAs were generated using AMV reverse transcriptase as above. The cDNAs were extracted with PCI, concentrated with a Centricon 30 microconcentrator, and the retentate was brought to 100 µl with TE to provide a 3′-RACE pool that was stored at 4 °C, and then used in the following amplification procedures.

The first procedure was designed to amplify nt 63–73, comprising the 3′ strands of the TψC and aminoacyl stems of previously unidentified nuclear DNA-encoded *M. senile* tRNAs. An *Eco*RI site-containing degenerate primer (TBTψC-1: 5′-CCGAATTCGGTTCRANYCC) was made, based on the highly conserved nt 52–62 tRNA sequences of standard tRNAs (Sprinzl et al. 2005). The reverse primer used in PCR amplification was TB17-3 (5′-GGAGATCT TTTTTTTTTTTTTTTTGG) that contains a 5′ *Bgl*II restriction site (underlined), 16 Ts and two 3′-terminal Gs to recognize the expected post-transcriptionally added 3′-terminal CCA. PCR amplification was carried out in a 100-µl reaction that included 1 µl of 3′ poly-A tailed cDNA, 25 pmol each of TBTψC-1 and TB-17-3, 0.2 mM each of dATP, dGTP, dCTP and dTTP and 2.5 U AmpliTaq™ DNA polymerase. For cycling, touchdown PCR (Don et al. 1991) was used as follows: 30 s at 94 °C, 30 s annealing temperature and 30 s at 72 °C with the annealing temperatures being 2 cycles at 48 °C, 2 cycles 46 °C, 2 cycles 44 °C, 2 cycles 42 °C, 2 cycles 40 °C, 2 cycles 38 °C then 25 cycles 37 °C. The double-stranded PCR products were digested with *Bgl*II and *Eco*RI, inserted into M13mp18, and resulting clones were sequenced.

For the second procedure, 5′-RACE analysis used a series of primers based on the 11 nt sequences obtained in

the first procedure to amplify tRNA sequences that are 5′ to nt 63. Each of these primers [designated TBAA (amino acid)-1 to TBAA-N, Fig. 3] comprised a *Pst*I site-containing sequence followed by a sequence complementary to one of the 11 nt sequences (tRNA sequence 63–73). Poly-C was added to the 3′ ends of cDNAs, from the 3′-RACE pool, using terminal deoxynucleotidyl transferase (Tdt, Amersham Life Science) in a 50-µl reaction containing 10 µl cDNA synthesized from poly-A tailed RNAs, 0.4 mM dCTP, and 30 U Tdt in the supplied reaction buffer and incubated for 5 min at 37 °C. Following extraction with PCI and Centricon 30 concentration, the retentate (designate the 5′-RACE pool) was made up to 100 µl with TE and stored at 4 °C. Amplification of specific 5′-extended sequences was achieved in 100 µl reactions containing 2 µl of the 5′-RACE pool, 0.2 mM each dATP, dCTP, dTTP and dGTP, 2.5 U AmpliTaq™ DNA polymerase (Perkin Elmer) and 25 pmol each of the tRNA-specific primer (TBAA-1 to TBAA-n) and primer TB17-4 (5′-CTGCGAATTCGGATCCTGTGGGGGGGG) which contains *Eco*RI and *Bam*HI sites (underlined) and recognizes the poly-C tail added with Tdt. Cycling used the touchdown PCR method described above to account for the uncertainty regarding the optimum annealing temperature of primer TB17-4. The double-stranded amplification products were cleaved with *Eco*RI and *Pst*I and cloned into *Eco*RI–*Pst*I-digested M13mp19. This insertion strategy placed the shorter (about 20 nt) poly-C tailed sequence closest to the M13 universal sequencing primer, which facilitated sequencing (the poly-A tails average about 50 nt). The resulting cloned cDNAs were sequenced using Sequenase™ (Amersham Life Science).

A third procedure designed to amplify previously unidentified tRNA 3′-end sequences, that may have been missed using oligonucleotide TBTψC-1, was also used. In this experiment, the tRNA-specific primers (TBAA-1 to

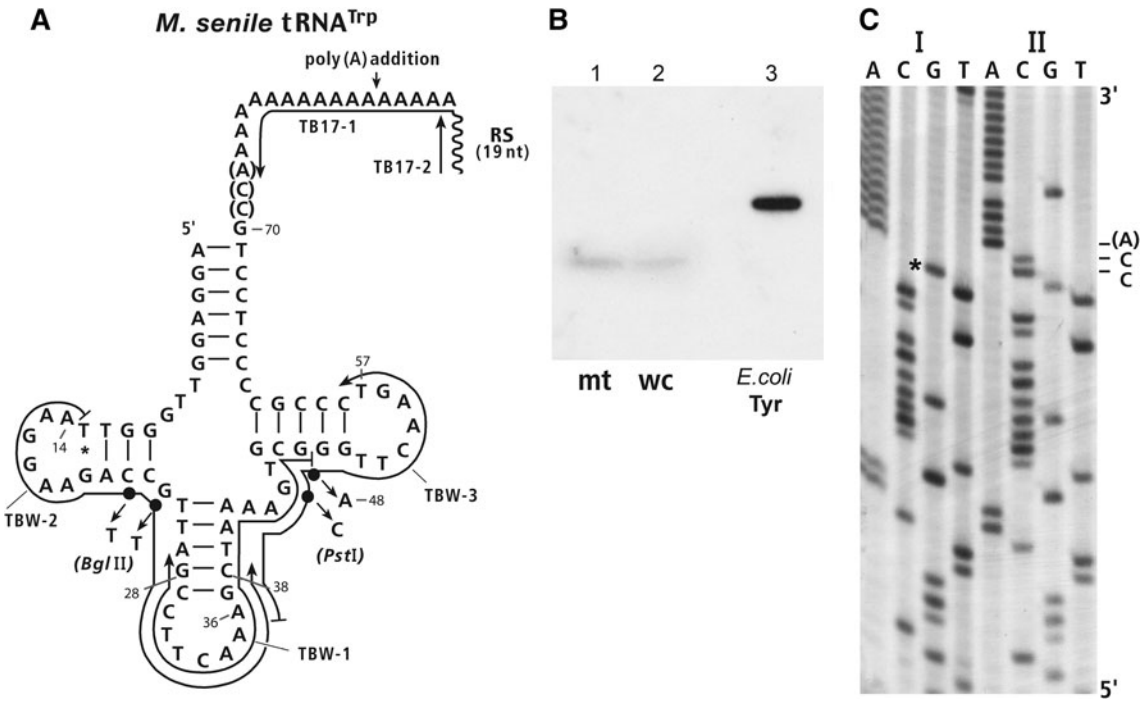

Fig. 3 Northern blot and 3′-RACE analysis of *M. senile* mt-tRNATrp. **a** The *M. senile* mt-tRNATrp gene shown in the presumed secondary structural form of the corresponding tRNA (nucleotides 1–70, Beagley et al. 1998) to which are added sequentially to the 3′ end the three nucleotides CCA (each in *parenthesis*) and 16 As representing polyadenylation with yeast poly(A) polymerase. The *three solid lines* TBW-1, TBW-2 and TBW-3 on the outside of the tRNA sequence identify segments to which oligonucleotide primers were made, with the *arrowheads* indicating the 5′–3′ direction. The *solid line* TB17-1 is a poly(A) primer with a 3′ *Eco*RI site-containing 19 nt (RS) segment. The *short solid line* TB17-2 is a primer complementary to the 3′ *Eco*RI site-containing sequence of TB17-1. The *solid dots* in primer TBW-2 indicate changes to the mt-tRNATrp gene sequence made to create a *Bgl*II site and the *solid dots* in primer TBW-3 indicate changes relative to the mt-tRNATrp gene sequence made to create a *Pst*1 site. **b** Northern blot hybridizations. *Lanes 1* and *2* contain RNA isolated from a mitochondrial fraction (*mt*) and from whole unfractionated cells (*wc*), and *lane 3* contains purified *E. coli* tRNATrp electrophoresed through a denaturing (15 % polyacrylamide, 8 M urea) gel. *Lanes 1* and *2* were probed with ^{32}P-labeled oligonucleotide TBW-1 and *lane 3* was probed with a ^{32}P-labeled oligonucleotide that is complementary to nucleotides 46–66 of *E. coli* tRNATyr. **c** Autoradiograph showing the products of sequencing of two clones of cDNAs of the 3′-end proximal regions of two mt-tRNATrp molecules. The sequence to the *right* (II) includes post-transcriptionally added CC (and presumably A) and the synthetically added poly(A) tail. The sequence to the *left* (I) also includes the synthetically added poly(A) tail but does not include post-transcriptionally added CC nucleotides

TBAA-n) were replaced in the 5′-RACE reactions by the non-specific primer TB17-3 (above). Reaction conditions and cycling were otherwise identical and this is designated a double-RACE amplification as both ends of the cDNA are unknown (except that TB17-3 selects for tRNA sequences by recognizing a 3′-terminal CCA). The double-stranded amplification products were cleaved with *Bgl*II and *Eco*RI and cloned into *Bgl*II–*Eco*RI-digested M13mp19 and sequenced as before.

Degenerate PCR amplification of the 3′-end sequence of *M. senile* cytoplasmic initiator tRNAi-Met

To determine the nucleotide sequence of the cytoplasmic (nuclear DNA-encoded) initiator methionine tRNA (tRNAi-Met) of *M. senile*, we used a degenerate oligonucleotide primer (TBiM-1: 5′-GCGCACYGGATCCGTGC TGGG; Fig. 4) which is a consensus based on the highly conserved nucleotides 10–31 of tRNAi-Met genes of human, mouse, *Xenopus laevis* and *Saccharomyces cerevisiae* (Fig. 4; Sprinzl et al. 2005). The two underlined nucleotides introduced mismatches that create a *Bam*HI restriction site for cloning. The PCR volume was 100 µl and included 1 µl poly-A-tailed cDNA (above), 25 pmol each of TBiM-1 and TB17-3, 0.2 mM each dATP, dCTP, dTTP and dGTP and 2.5 U AmpliTaq^{TM} DNA polymerase. Cycling for this reaction was 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C for 30 cycles. The resulting double-stranded PCR product was digested with *Bgl*II and *Bam*HI and cloned into *Bam*HI-digested M13mp19 for sequencing.

Amplification of nuclear DNA-encoded tRNA genes

High molecular weight DNA was isolated from *M. senile* using protocol II of the Stratagene DNA extraction kit: Extraction from whole tissue. A DNA-based genomic library was created by digesting to completion 1 µg DNA with *Sau*3AI and ligating the resulting fragments into *Bam*HI-digested M13mp19 double-stranded DNA (100 ng). The chimeric DNA was extracted once with PCI and desalted using a Centricon 100 microconcentrator. The retentate was made up to 100 µl with TE and the library was stored at 4 °C. From this library, DNA sequences were amplified using tRNA-specific oligonucleotide primers TBAA-N (Fig. 4B) to anchor one end of the PCR and the universal M13 sequencing primer (New England Biolabs) to anchor the other end. PCR included 1 µl of the library DNA, 0.2 mM each dATP, dCTP, dTTP and dGTP, 25 pmol each oligonucleotide primer and 2.5 U Amplitaq^{TM} DNA polymerase. The sequence of the oligonucleotide primer used to amplify the tRNAIle DNA sequence (TB-Ile) was 5′-GGTCTAGATGGTCCGTATGGGGC (underlined nucleotides comprise an added *Xba*I site) and the annealing temperature was 40 °C. The sequence of the oligonucleotide primer used to amplify the tRNAVal DNA sequence (TB-Val) was 5′-GGTCTAGATGTTTCCGCCC GGGC and the annealing temperature was 40 °C. The sequence of the oligonucleotide primer used to amplify the cytoplasmic initiator tRNAi-Met DNA sequence (TB1 M-2) was 5′-GGTCTAGATGCAGAGCGAGGTTTCGATC [that includes an *Xba*I site (underlined)] and the annealing temperature was 50 °C. Amplification products were cloned into M13mp18 by cleaving with *Xba*I and *Eco*RI (amplified from the M13mp19 poly-linker cloning site) and sequenced.

Preparation of mechanically damaged mitochondria

M. senile mitochondria were purified by isopycnic sucrose (1.0–2.0 M) centrifugation (Rickwood et al. 1987). Half of the sucrose gradient-purified mitochondria were passed three times through an 18-gauge needle and these mitochondria and the remaining half of the sucrose gradient-purified mitochondria were separately sedimented through 20–60 % percoll gradients. The mitochondria that had not been passed through a needle formed a single band and are designated undamaged. In contrast, the mitochondria which were passed through a needle formed two distinct bands of approximately equal intensity, one at the same position as undamaged mitochondria, and the other (presumably damaged) at a higher density. This change in density is characteristic of disruption of the outer mitochondrial membrane (Rickwood et al. 1987). From these two mitochondrial fractions, 40–160 nt fraction of RNA was prepared as before.

Northern analysis of tRNA cellular distribution

Cytoplasmic, mitochondrial and damaged mitochondrial RNAs (1 µg each) were electrophoresed in a denaturing (15 % polyacrylamide, 8 M urea) gel, electroblotted to nylon membranes and probed with ^{32}P-labeled oligonucleotides as has been previously described (Okimoto et al. 1990). The sequences of the oligonucleotides used to probe for mt-tRNAf-Met (TMB-1) and mt-tRNATrp (TBW-1) were 5′-TGTAGAGGCAGGAATTGAA and 5′-CGCACT TTTAGCTTTGAAGGC, respectively. The sequences of the oligonucleotide probes for nuclear-encoded tRNAVal and tRNAi-Met were 5′-GTGATAACCACTACACTACG and 5′-GCAGAGCGAGGTTTCGATC, respectively.

Results

M. senile mt-tRNAs

We carried out experiments to examine whether *M. senile* mitochondria contain functional transcripts of the

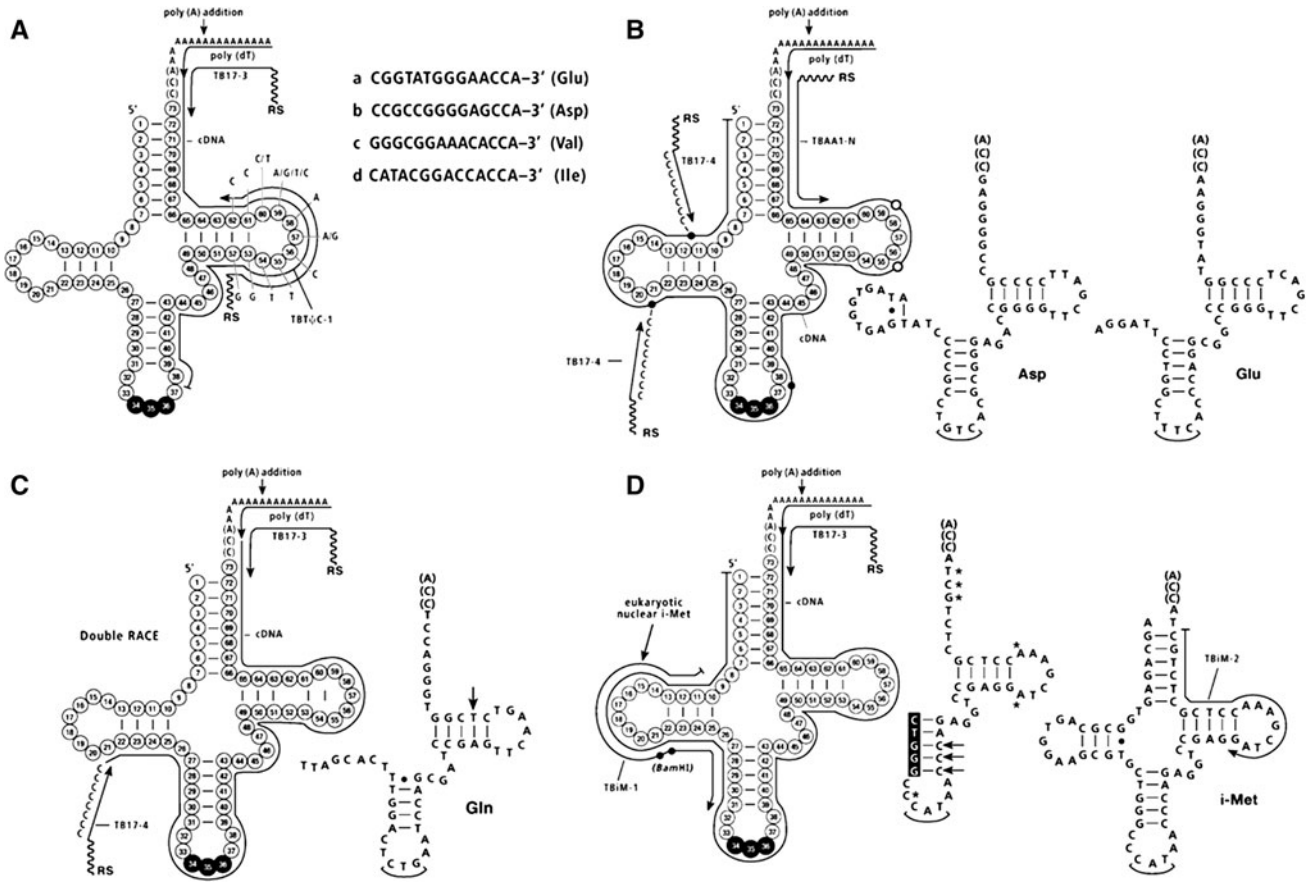

Fig. 4 Reverse transcriptase-PCR experiments carried out to obtain sequences of *M. senile* nuclear DNA-encoded tRNAs. **A–D** A nucleotide numbered standard tRNA (Sprinzl et al. 2005) is shown that has been 3′ polyadenylated with yeast poly-A polymerase. Anticodon nucleotides (#34, #35, #36) are shown as *reverse numbers*. The expected post-transcriptionally added CCA nucleotides on each tRNA are *bracketed*. In each experiment, cDNA synthesis was primed with poly-dT. *Lines* outside the tRNAs represent cDNAs (labeled) and oligonucleotide primers, the 5′–3′ directions of which are shown by *arrowheads*. The partial, specific tRNA sequences obtained from these experiments are shown to the *right* of each schematic tRNA, either as a linear sequence (**A**) or as sequences folded into presumed secondary structural configurations (**B–D**). **A** A scheme used to obtain sequences of nucleotides 63–73 of unknown tRNAs. Oligonucleotide primers used, shown as *solid lines* outside the sequence, are: TB17-3 a 5′-restriction site (RS)-containing sequence added to 16 Ts and GG; TBTψC-1, a 5′-restriction site-containing sequence added to an 11-nt degenerate sequence (5′-GGTTCPuANPyCC, shown outside the *solid line*) that matches the consensus sequence of the distal two nucleotide pairs of the TψC stem and the TψC loop of the majority of standard tRNAs (Sprinzl et al. 2005). Four of the 29 unique, 11 nt sequences obtained, that were used in further experiments are shown to the *right* (*a–d*, and labeled to the *right* with the identification of each determined in later experiments). **B** A scheme employing information gained in **A** to obtain tRNA sequences 5′ to nucleotide 63. The *solid dots* on the cDNA indicate positions at which cDNA synthesis was most frequently terminated. Poly-C was added to the 3′ ends of cDNAs using terminal nucleotidyl transferase. Primers TBAA-1-N are sequences comprising a 5′-restriction site (RS) and the 4 different 11 nt sequences obtained in **A**. TB17-4 is a 5′-restriction site-containing sequence (RS) followed by eight Gs. Two partial tRNA sequences obtained with anticodons identifying them as tRNAAsp and tRNAGlu are shown to the *right*. **c** A scheme (double RACE) to obtain tRNA sequences irrespective of whether the TψC arm sequence corresponds to the degenerate sequence shown in **A**. A partial tRNA sequence obtained with an anticodon indicating it to be tRNAGln is shown to the *right*. The *arrow* indicates an A52–T62 pair that would not be recognized by primer TBTψC-1 (see **A**). **D** An experiment designed to obtain the sequence of tRNAi-Met. Primer TBiM-1 is a consensus sequence based on nucleotides 10–31 of nuclear tRNAi-Met genes of four eukaryotes. The *solid dots* indicate two consensus sequence nucleotides (#21 and #22) that have been changed to create a *Bam*HI site. The partial *M. senile* tRNAi-Met sequence obtained is shown to the *right*. The reverse lettered five nucleotides originate from the primer. *Asterisks* identify nucleotides (#33, #54, #60, #70, #71 and #72) that are characteristic of all known eukaryotic tRNAi-Mets, and *arrows* (nt 39–41) indicate 3 Cs at the base of the anticodon stem that are characteristic of tRNA initiator methionine and some formyl-methionine specifying tRNAs (RajBhandary and Chow 1995). A primer, TBiM-2, complementary to nt 54–71 of the partial cDNA-tRNAi-Met sequence was used to obtain the nuclear DNA-encoded gene sequence, shown to the *far right*

mt-tRNAf-Met and mt-tRNATrp genes. Northern blot analyses were performed on mt-RNA and wc-RNA (Fig. 1) using mt-tRNAf-Met-specific and mt-tRNATrp-specific oligonucleotide primers (Figs. 2a, 3a). In each of the resulting autoradiographs, a single band of the approximate sizes expected for mt-tRNAf-Met and mt-tRNATrp was

observed (Figs. 2b, 3b). For each tRNA probe, the band was darker in the mt-tRNA-containing lane than in the wc-RNA-containing lane. These data indicate that RNA molecules of the sequences and approximate sizes expected for tRNAs encoded by the mt-tRNAf-Met and mt-tRNATrp genes are present in *M. senile* mitochondria.

To determine whether CCA is post-transcriptionally added to the 3′ ends of each of the two mtDNA-encoded tRNAs, as would be expected if they are functional, we did the following: *M. senile* mt-RNAs were polyadenylated, then used in an RT-PCR containing an mt-tRNAf-Met-specific sense primer (Fig. 2a). Sixteen clones of the PCR amplification product were obtained and sequenced. All were mt-tRNAf-Met sequences. Eight of these sequences contained all of the expected tRNA sequence 3′ to the gene-specific primer, that is, nucleotides 41–71, followed by CC and a poly-A sequence (Fig. 2c). This indicates that to at least half of the mt-tRNAf-Met molecules, CC nucleotides, and possibly an A nucleotide, but not any of the three other nucleotides, are added following transcription: a post-transcriptionally added A would not be distinguishable from the synthetically added A. In the remaining eight cloned sequences, a poly-A sequence followed various nucleotides of the 3′-end proximal region of the encoded tRNA sequence. It seems more likely that these sequences resulted from degradation of the tRNA 3′-end during the experimental procedures than from incomplete transcription.

A similar experiment was carried out for mt-tRNATrp. In this case, the RT-PCR contained an mt-tRNATrp-specific sense primer (Fig. 3a). Twelve clones from the amplified product were sequenced. All 12 were $tRNA^{Trp}$ sequences, but all lacked a 3′-terminal CC(A). In three and nine, a poly-A sequence followed the 3′-terminal nucleotide and the 3′-sub-terminal nucleotide predicted by the mt-tRNA gene, respectively. Because the gene-specific primer (TBW-2) used in PCR amplifications recognizes sequences 3′ to the nucleotide following the anticodon [nt 35 in Fig. 3a, nt 37 in standard tRNAs (Sprinzl et al. 2005)] which is a modified A in many mt-tRNAs as well as cytoplasmic tRNAs (Randerath et al. 1981; Roe et al. 1982; Dirheimer et al. 1995; Helm et al. 1998), it seems plausible that the TBW-2 primer had selected tRNAs in which A_{35} modification, and perhaps CCA addition, had yet to occur. To test this possibility, we repeated the PCR experiment using a primer (TBW-3) that matches nucleotides 36–57 of the mt-tRNATrp gene (Fig. 3a). Of 15 mt-tRNATrp sequences recovered, one had a 3′-terminal CC followed by poly-A (Fig. 3c, II). In the other 14 sequences, poly-A followed the 3′-terminal nucleotide (Fig. 3c, I), the sub-terminal nucleotide and an internal nucleotide in 9, 4, and 1, respectively. These data suggest that of the tRNATrp molecules present in *M. senile* mitochondria, only a small fraction contain a post-transcriptionally added CC(A). At the same time, it remains tenable that nucleotide modifications specific to mature mt-tRNATrp molecules resulted in selection against fully processed tRNAs by the mt-tRNATrp gene-specific primers used (TBW-2 and TBW-3, Fig. 3a). Data from both of the above experiments again suggest degeneration at the tRNA 3′ end during processing.

M. senile nuclear-encoded tRNA sequences

The primary nucleotide sequence of the TψC loop (nucleotides 54–60) of eukaryotic nuclear DNA-encoded tRNA genes is highly conserved (5′-TTCRANY) and the loop is almost always preceded by two G–C base pairs, G52–C62 and G53–C61 (Sprinzl et al. 2005; Juhling et al. 2009). This information was used to design a degenerate oligonucleotide primer that could be used to PCR amplify the 3′ terminal of 11 nucleotide-encoded sequences of *M. senile* nuclear DNA-encoded tRNAs (Fig. 4A). *M. senile* mt-RNAs were polyadenylated, then used in an RT-PCR containing an antisense primer that recognizes the 3′-CCA of proposed tRNAs (Fig. 4A). The double-stranded PCR products were cloned and sequenced. Of the cloned sequences derived from mt-RNAs and wc-RNAs, 22 (of 23 total) and 18 (of 21 total) were of the size [11 nt + CC(A)] expected for tRNA sequences between the 3′ end of the degenerate primer and the 3′ end of the tRNA (nucleotides 63–73 + CCA, Fig. 4A). Among the 22 mt-RNA-derived, 11 nt sequences, there were 16 different kinds (that is a small number of sequences were represented multiple times). Of the 18 wc-RNA-derived, 11 nt sequences, there were 17 different kinds but 4 of these were also recovered from mt-RNA. Therefore, a total of 29 different sequences were obtained from mt-RNA and wc-RNA.

To determine the sequences that extend 5′ to 4 of the above-mentioned 11 nt sequences (Fig. 4A; a–d), 5′-RACE experiments were carried out (Fig. 4B). CDNAs synthesized from 3′ polyadenylated wc-RNAs were 3′ poly-C tailed, then used in PCR amplification methods with a primer that includes a restriction site-containing sequence followed by 8 Gs, and with one of four primers (TBAA1-N, Fig. 4B) each comprising a restriction site-containing sequence followed by GG, and a sequence complementary to one of the four 11 nt sequences (a–d) shown in Fig. 4A. The PCR products were cloned and sequenced. The four sequences obtained were 52, 32, 63 and 36 nt and each could be folded into a partial tRNA. The variable, less than complete tRNA sequence lengths may have resulted from inhibition of reverse transcriptase by different modified nucleotides (Limbach et al. 1994) in the four tRNA sequences recognized by the primers. The 32 and 36 nt sequences stopped short of the anticodon. However, the 63 and 52 nt sequences included the anticodons 5′-TTC and

5′-GTC, expected for tRNAAsp and tRNAGlu (Fig. 4B). Each of the four 11 nt presumptive tRNA-specific primers used in this experiment were ^{32}P-end labeled and used to probe Northern blots of mt-RNA and wc-RNA. In each lane, primers hybridized to a single band of the expected size (about 70 nt) for tRNAs, and in each case, the signal was stronger for wc-RNA than for mt-RNA (data not shown).

To determine the nucleotide sequences of *M. senile* nuclear DNA-encoded tRNAs with TψC arm sequences that might not have been recognized by the degenerate TBTψC-1 oligonucleotide, we carried out PCR amplification using double-tailed cDNAs [designated a double-RACE experiment (Fig. 4C)]. The double-stranded PCR products were cloned and sequenced. Twenty different sequences were obtained. Ten of these were extensions of those identified in experiments using the degenerate TBTψC-1 primer (Fig. 4A) and ten were new sequences. However, 19 of the 20 sequences were in the range of 15–17 nt. Again, it seems likely that inhibition of reverse transcriptase by modified nucleotides contributed to the short length of these sequences. The one exception was a new sequence of 45 nt that could be folded into a partial tRNAGln (anticodon 5′-CTG, Fig. 4C). The A52–U62 base pair present in this sequence may have impaired pairing of the TBTψC-1 primer in the experiment represented in Fig. 4A. An oligonucleotide recognizing the partial putative tRNAGln sequence hybridized to a single band of tRNA size in a Northern blot of wc-RNA (not shown).

M. senile cytoplasmic initiator tRNAi-Met

One of the most highly conserved tRNAs in eukaryotic cells is the nuclear DNA-encoded initiator tRNAi-Met (RajBhandary and Chow 1995). To determine the nucleotide sequence of the *M. senile* tRNAi-Met, we performed a 3′-RACE amplification using a degenerate oligonucleotide (primer TBiM-1, Fig. 4D) based on the consensus sequence of nucleotides 10–31 of 4 eukaryotic cytoplasmic initiator tRNAs (*S. cerevisiae*, *X. laevis*, mouse and human). The PCR product was cloned and sequenced, and found to comprise a 42-nt sequence that could be folded into a partial tRNA with a 5′-CAT anticodon, 3 Cs (nucleotides 39, 40, 41) at the base of the anticodon stem and 6 nucleotides (C_{33}, A_{54}, A_{60}, G_{70}, C_{71}, and T_{72}) that are characteristic of a eukaryotic tRNAi-Met (Fig. 4D) (RajBhandary and Chow 1995).

Cloning of *M. senile* nuclear DNA-encoded tRNA genes

To determine the identity of the tRNAs represented by 2 of the 11 nt sequences (c and d) shown in Fig. 4A, and to obtain the remainder of the tRNAi-Met sequence, a PCR-based genomic cloning strategy was used to locate the genes for these tRNAs. High molecular weight *M. senile* DNA was digested to completion with *Sau*3AI and the resulting restriction fragments were ligated into *Bam*HI-cleaved bacteriophage M13 DNA. From this nuclear DNA library, the gene sequence for each tRNA was amplified in three different PCR using, as primers, restriction site-containing oligonucleotides complementary to each of the two 11 nt sequences (c and d, Fig. 4A) and to the known 3′ portion of tRNAi-Met (primer TBiM-2, Fig. 4D), and the universal M13 sequencing primer. The complete tRNAi-Met gene sequence and a short upstream segment were obtained (Fig. 4D). This gene has 72 nt and has an identity to the mtDNA-encoded tRNAf-Met gene (Fig. 2a) of only

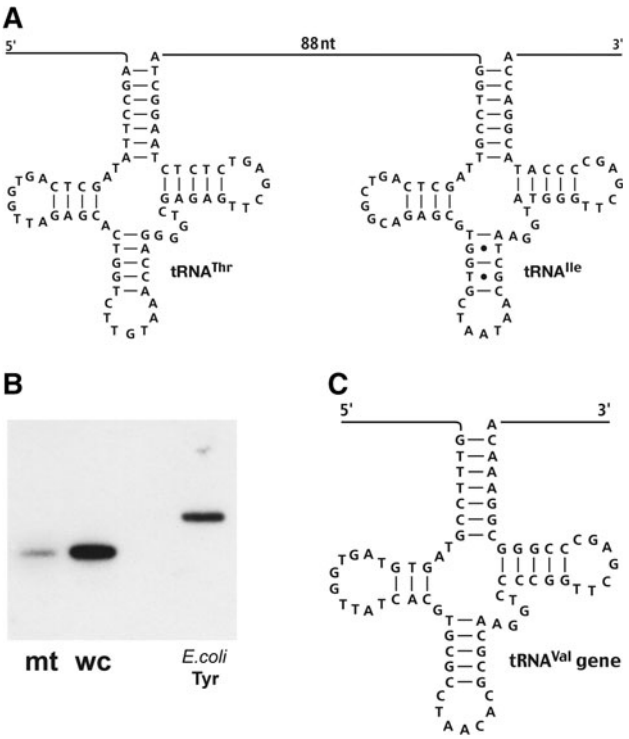

Fig. 5 Nuclear DNA-encoded tRNA genes. *M. senile* nuclear DNA sequences containing genes for tRNAThr and tRNAIle (**a**) and for tRNAVal (**c**) shown in their presumed secondary structure configuration. The 3′-terminal 11 nt sequences for tRNAIle and tRNAVal were obtained from the results of a 3′-RACE experiment, using primer TBTψC-1 (Fig. 4A). Primers complementary to these 11 nt sequences were used with the M13 universal sequencing primer to obtain by PCR the remainder of the sequences shown (including the entire tRNAThr gene) from an M13 nuclear DNA library of *Sau*3AI fragments. **b** Northern blot. In the autoradiograph, the *three lanes* contain *M. senile* mt-RNA (mt), whole cell RNA (wc) and *E. coli* tRNATyr (85 nt), separated by electrophoreses through a denaturing (15 % polyacrylamide, 8 M urea) gel. *Lanes mt* and *wc* were probed with a ^{32}P-labeled oligonucleotide that are complementary to the 11 nt sequence for tRNAIle shown in Fig. 4A. *Lane E.coli Tyr* was probed with a ^{32}P-labeled oligonucleotide complementary to nt 46–66 of *E. coli* tRNATyr

55.4 %. Also obtained were sequences that could be folded into tRNA genes with 5′-AAT (isoleucine-specifying) and 5′-AAC (valine-specifying) anticodons (Fig. 5a, c). Additionally, 88 nt upstream of the 5′ end of the tRNAIle gene was located a sequence whose RNA product could be folded into the secondary structure of a tRNAThr (Fig. 5a).

Distribution of nuclear DNA-encoded tRNAs in the cytoplasm and in mitochondria

Northern blot experiments were carried out to examine the cellular distribution of *M. senile* mtDNA-encoded tRNAf-Met and the nuclear DNA-encoded tRNAVal and tRNAi-Met (Fig. 6). Transfer RNA-specific oligonucleotides representing the mt-tRNAf-Met gene and the nuclear tRNAVal and tRNAi-Met genes were hybridized to blots of small RNAs prepared from a cytoplasmic fraction from which mitochondria had been removed by differential centrifugation, from a percoll gradient-purified mitochondrial fraction, and from a percoll gradient-purified fraction of mitochondria that had been mechanically damaged to the point of altering their relative density in a percoll gradient. As such a change in density is characteristic of mitochondria from which the outer membrane has been removed (Rickwood et al. 1987), it was reasoned that cytoplasmic tRNAs attached to the outer mitochondrial membrane would be eliminated. The results of this experiment are shown in Fig. 6. All three tRNAs were detected in each of the three RNA preparations. In the lanes containing cytoplasmic RNA, each of the three probes hybridized to a single band of the size expected for each mature tRNA. However, relative to the tRNAVal and tRNAi-Met bands (Fig. 6b, c), the mt-tRNAf-Met was much lighter (Fig. 6a). In each of the two mitochondrial fractions, each tRNA-specific oligonucleotide recognized two distinct bands, and in all cases, the upper is darker and of the size expected for the mature (CCA-containing) tRNAs. In each case, the lower band is at a position expected for molecules approximately three nucleotides smaller than those in the upper band, consistent with this band comprising tRNAs that lack the 3′-terminal CCA. It is of note here that 3′-RACE analysis of the *M. senile* mt-tRNAf-Met molecules showed that half of these tRNAs lacked the 3′-terminal CCA. It was surprising to find that the *M. senile* cytoplasmic initiator methionine tRNA occurs in high amounts in the mt-RNA fractions, as it is expected that this tRNA would not be used to initiate mitochondrial translation.

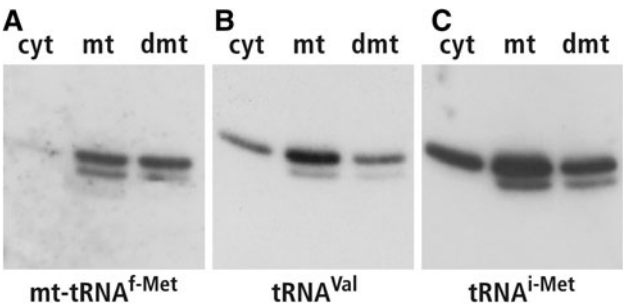

Fig. 6 Northern blot analyses to examine the cellular distribution of *M. senile* mtDNA-encoded tRNAf-Met and nuclear DNA-encoded tRNAVal and tRNAi-Met. Small RNAs prepared from a cytoplasmic fraction from which mitochondria had been removed (*cyt*), percoll gradient-purified undamaged mitochondria (*mt*), and damaged mitochondria (*dmt*) (1 µg each) were electrophoresed in a denaturing (15 % polyacrylamide, 8 M urea) gel, electroblotted to nylon membranes and probed with ^{32}P-end labeled oligonucleotides specific for the three tRNAs as shown

Discussion

The aim of the present study was to obtain experimental evidence regarding the possible participation in mt-protein synthesis of the sea anemone, *M. senile* of the only two tRNAs (tRNAf-Met and tRNATrp) encoded in the mt-genome of this organism, and of tRNAs transcribed from the *M. senile* nuclear genome. The results we obtained clearly establish that RNAs of the size and sequence predicted by the mt-tRNAf-Met and mt-tRNATrp genes occur in mitochondria. Further, the finding of both mt-tRNAf-Met and mt-tRNATrp with CC (most probably followed by an A, but never by another nucleotide) at the 3′ terminus, the post-transcriptional modification expected for them to be charged with the respective amino acid, greatly strengthens the likelihood that these tRNAs are functional in mt-protein synthesis. Also, direct evidence was obtained indicating that a fraction of at least two nuclear DNA-encoded tRNAs (tRNAi-Met and tRNAVal) are imported into mitochondria, supporting the contention that these tRNAs are essential elements in the synthesis of mt-proteins.

The very low frequency of mt-tRNATrp molecules with a CC(A) detected could have resulted from interference with the PCR of post-transcriptionally modified nucleotides in CC(A)-containing tRNAs. However, there may, in fact, be a low frequency of post-transcriptional addition of CC(A) to mt-tRNATrp molecules which would correlate with the much lower use of TGA codons (14) than TGG codons (88) in the 14 *M. senile* mt-protein genes. The ratio of TGA to TGG codons (1:6.3) is inversely correlated to the overall ratio of other codons ending in A and G (1.7:1) in the *M. senile* mt-protein genes (Beagley et al. 1998). A similar finding was reported for the mtDNA of the octocoral *Sarcophyton glaucum*: The ratio of TGA to TGG codons is 1:14.0, and the ratio of codons ending in A and G is 2.6:1 (Pont-Kingdon et al. 1998). In contrast, in bilaterian mt-protein gene sets, the ratio of TGA to TGG codons is directly correlated with the ratio of other codons ending

in A and G. The ratios of TGA:TGG codons and of A:G endings of other codons in some bilateria are: mouse, 13.9:1/14.6:1 (Bibb et al. 1981); *Drosophila yakuba*, 16.0:1/15.7:1 (Clary and Wolstenholme 1985); *C. elegans*, 6.1:1/4.1:1; *A. suum*, 0.6:1/0.5:1 (Okimoto et al. 1992).

Data from Northern blot analyses using an mt-tRNAf-Met-specific primer indicated that this tRNA is essentially confined to mitochondria, consistent with the expectation that it is utilized specifically to initiate the synthesis of mt-proteins. In each of the two mt-RNA fractions, the mt-tRNAf-Met-specific primer recognized two size classes of molecule, one at the position expected for a mature tRNA and one of lesser frequency at a position expected for a molecule three nucleotides shorter. This observation is consistent with the direct evidence obtained by sequencing mt-tRNAf-Met that a fraction of these molecules has a CC(A) post-transcriptionally added to their 3′ ends.

The presence of nuclear DNA-encoded tRNAVal and tRNAi-Met in both the mitochondria and the cytoplasm is clearly indicated in the Northern blots, suggesting that at least these two tRNAs function in both cytoplasmic and mt-protein synthesis. However, as was observed for the mt-tRNAf-Met primer, both the tRNAVal primer and the tRNAi-Met primer recognized two bands in each of the mitochondrion-derived RNAs, consistent with the presence of tRNAVal and tRNAi-Met molecules with and without post-transcriptionally added CCA. Again, the frequencies of the smaller molecules appeared to be less than those of the larger molecules. In contrast, each of the tRNAVal and the tRNAi-Met primers recognized only a single band in the cytoplasmically derived RNAs, at the same position as that for the larger bands in RNAs from mitochondria. Taken together, these observations indicate that although it is expected that CCA is added to nuclear DNA-encoded tRNAs immediately following transcription (Schneider 2011), tRNAVal and tRNAi-Met molecules destined for importation have CCA added only after they have entered mitochondria. However, the substantial fraction of tRNA-Val and tRNAi-Met molecules that in mitochondria appear to lack CCA may indicate a lag in transportation of these tRNAs across the mitochondrial membrane, or that CCA addition is much less efficient in mitochondria than in the cytoplasm. Alternatively, these data are consistent with the interpretation that tRNAs with and without CCA may be transported into mitochondria.

Importation of nuclear DNA-encoded tRNAs into mitochondria has been extensively studied in a variety of organisms (reviewed in Schneider 2011), providing evidence for two general mechanisms. In one, examined in greatest detail for a tRNALys encoded in nuclear DNA of the yeast *S. cerevisiae*, and defined cytosolic factors are involved, the tRNA is linked to a lysosyl-tRNA synthetase and the complex is co-imported along the mitochondrial import pathway. In the second mechanism, found to operate in plants and protozoa, tRNAs are directly imported into mitochondria with much less or no cytosolic factors involvement. At this time, there is no direct evidence regarding the mechanism of importation of nuclear DNA-encoded tRNAs into cnidarian mitochondria. However, the detection in nuclear DNA of the hexacoral *Nematostella vectensis* of genes for aminoacyl-tRNA synthetases (Haen et al. 2010) that include mitochondrion-targeting sequences leaves open the possibility that a co-importation mechanism operates in Cnidaria.

From the bioinformatic analysis of DNA of *N. vectensis*, Haen et al. (2010) postulated the occurrence of a methionyl-tRNA synthetase expected to have a bifunctional role in cytoplasmic protein synthesis and mt-protein synthesis. However, a methionyl-tRNA synthetase expected to be specific to mtDNA-encoded methionine was not detected. In view of our finding of *M. senile* mt-tRNAf-Met molecules with post-transcriptionally added CC(A), it seems plausible that the single imported methionyl-tRNA synthetase is responsible for the charging of formylated tRNAMet molecules involved in the initiation of mt-protein synthesis as well as tRNAMet molecules specifying internal methionines. It remains unclear what role the imported nuclear DNA-encoded tRNAi-Met molecules we detected in *M. senile* mitochondria might play in mt-protein synthesis. One possibility is that this tRNA participates in decoding internal AUG codons in transcripts of mt-protein genes. However, this would not be consistent with the situation in either eukaryotic cytoplasmic protein synthesis or prokaryotic protein synthesis, where in each case a tRNAMet (elongator tRNAMet) with a considerably different primary structure (sequence) than the corresponding tRNAi-Met is used to decode internal AUG codons (RajBhandary and Chow 1995; Beagley et al. 1995).

As noted previously (Beagley et al. 1998), the retention of genes for tRNAf-Met and tRNATrp in the *M. senile* mt-genome is likely related to functions specific to these tRNAs: the initiation of metazoan mt-protein synthesis by formyl-methionine rather than methionine (Chomyn et al. 1981), and the need for a tRNA that recognizes TGA as tryptophan-specifying. Although the 5′-UCA anticodon of the mt-tRNATrp would be expected to recognize both 5′-UGA and 5′-UGG codons (Beagley et al. 1998), it is clearly worthwhile to consider whether in translation of *M. senile* mt-protein genes, 5′-UGG codons might be decoded by a tRNA transcribed from nuclear DNA that is also utilized to recognize 5′-UGG codons in transcripts of nuclear protein genes. This hypothesis is supported by the bioinformatic data of Haen et al. (2010) that *N. vectensis* nuclear DNA encodes two tryptophyl-tRNA synthetases, one specific for the mtDNA-encoded tRNATrp and one that is specific for a nuclear-encoded tRNATrp.

A paucity of tRNA genes appears to be a common feature of Cnidarian mtDNAs. Genes for only tRNAf-Met and tRNATrp occur in mtDNAs of species of the subclass Hexacorallia of Anthozoa, Hydrozoa, and Scyphozoa so far examined (Beagley et al. 1995, 1998; Pont-Kingdon et al. 2000; van Oppen et al. 2002; Shao et al. 2006; Kayal and Lavrov 2008). However, mtDNAs of species of the subclass Octocoralia of Anthozoa contain only tRNAf-Met. (Beagley et al. 1995; Beaton et al. 1998; Medina et al. 2006; Fukami and Knowlton 2005). Clearly, for protein synthesis to occur in *M. senile* mitochondria, all but two tRNAs must be imported from the cytoplasm. The data presented in this paper suggest that at least some of the *M. senile* nuclear DNA-encoded tRNAs are utilized in both cytoplasmic and mt-protein synthesis. This is in line with previous findings regarding angiosperm plants for which there is evidence for the common use of some nuclear DNA-encoded tRNAs in both the cytoplasm and the mitochondria (Marechal-Drouard et al. 1988), and for the protozoans *L. tarentolae* and *T. brucei* in which all tRNAs used in mt-protein synthesis are nuclear DNA-encoded, and are also used in cytoplasmic protein synthesis (Simpson et al. 1989; Hancock and Hajduk 1990). However, it has been shown that in *T. brucei*, the nuclear DNA-encoded tRNAi-Met is excluded from importation into mitochondria (Esseiva et al. 2004) which contrasts with our conclusion regarding this tRNA in *M. senile.*

Both *M. senile* mt-tRNAf-Met and mt-tRNATrp have primary and secondary structural properties characteristic of *M. senile* nuclear DNA-encoded tRNAs (and other standard tRNAs) rather than the reduced structural characteristics of bilaterian mt-tRNAs. This is in line with all of the tRNAs within a mitochondrion having to interact with a single ribosome type. Interestingly in this regard, it has been found that the predicted secondary structures of *M. senile* mt-s-rRNA and mt-l-rRNA both more closely resemble the corresponding rRNAs of prokaryotes than the mt-rRNA of any bilaterian species (Pont-Kingdon et al. 1994, 2000; Beagley et al. 1998).

The data reported from the present study provide new information regarding the mechanism of mt-protein synthesis in a cnidarian. Clearly, an analysis of the amino acids associated with isolated tRNAs is needed to confirm the suggested involvement of the two mtDNA-encoded tRNAs and of the nuclear DNA-encoded tRNAs, particularly tRNAi-Met. A continuation of the presently reported study can be expected to fully define the identity of all nuclear DNA-encoded tRNAs that are imported into mitochondria, and together with the bioinformatic information regarding importation of aminoacyl-tRNA synthetases (Haen et al. 2010) would greatly extend our understanding of how mt-proteins are synthesized in Cnidaria.

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References

Abascal F, Posada D, Zardoya R (2012) The evolution of the mitochondrial genetic code in arthropods revisited. Mitochondr DNA 23:84–91

Adshya S (2008) *Leishmania* mitochondrial tRNA importers. Int J Biochem Cell Biol 40:2681–2685

Alfonzo JD, Soll D (2009) Mitochondrial tRNA import—the challenge to understand has just begun. Biol Chem 390:717–722

Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Barrell BG, Bankier AT, Drouin J (1979) A different genetic code in human mitochondria. Nature 282:189–194

Barrell BG, Anderson S, Bankier AT, de Bruijn MHL, Chen E, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1980) Different pattern of codon recognition by mammalian mitochondrial tRNAs. Proc Natl Acad Sci USA 77:3164–3166

Beagley CT, Macfarlane JL, Pont-Kingdon GA, Okimoto R, Okada NA, Wolstenholme DR (1995) Mitochondrial genomes of Anthozoa (Cnidaria). In: Palmieri F, Papa P, Saccone C, Gadaleta N (eds) Progress in cell research: symposium on "30 years of progress in mitochondrial bioenergetics and molecular biology". Elsevier Science BV, Amsterdam, pp 149–153

Beagley CT, Okada NA, Wolstenholme DR (1996) Two mitochondrial group I introns in a metazoan, the sea anemone *Metridium senile*: one intron contains genes for subunits 1 and 3 of NADH dehydrogenase. Proc Natl Acad Sci USA 93:5619–5623

Beagley CT, Okimoto R, Wolstenholme DR (1998) The mitochondrial genome of the sea anemone, *Metridium senile* (Cnidaria): introns, a paucity of tRNA genes, and a near standard genetic code. Genetics 148:1091–1108

Beagley CT, Okimoto R, Wolstenholme DR (1999) *Mytilus* mitochondrial DNA contains a functional gene for a $tRNA^{Ser}$(UCN) with a dihydrouridine arm replacement loop, and a pseudo-$tRNA^{Ser}$(UCN) gene. Genetics 152:641–652

Beaton MJ, Roger AJ, Calvalier-Smith T (1998) Sequence analysis of the mitochondrial genome of *Sarcophyton glaucum*: conserved gene order among octocorals. J Mol Evol 47:697–708

Bernt M, Braband A, Schierwater B, Peter F. Stadler PF (2012) Genetic aspects of mitochondrial genome evolution. Mol Phylogenet Evol, in press

Bibb MH, van Etten RA, Wright CT, Walberg MW, Clayton DA (1981) Sequence and gene organization of mouse mitochondrial DNA. Cell 26:167–180

Boore JL (1999) Animal mitochondrial genomes. Nucl Acids Res 27:1767–1780

Brown TA, Cecconi C, Tkachuk AN, Bustamante C, Clayton DA (2005) Replication of mitochondrial DNA occurs by strand

displacement with alternative light-strand origins, not via a strand-coupled mechanism. Gene Dev 19:2466–2476

Burger G, Plante I, Lonergan KM, Gray MW (1995) The mitochondrial DNA of the amoeboid protozoon, *Acanthamoeba castellanii*: complete sequence, gene content and genome organization. J Mol Biol 245:522–537

Chomyn A, Hunkapillar MW, Attardi G (1981) Alignment of the amino terminal amino acid sequence of human cytochrome c oxidase subunits I and II with the sequence of their putative mRNAs. Nucl Acids Res 9:867–877

Clary DO, Wolstenholme DR (1985) The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization and genetic code. J Mol Evol 22:252–271

De Bruijn MHL, Schreier PH, Eperon IC, Barrell BG, Chen EY, Armstrong PW, Wong JFH, Roe BA (1980) A mammalian mitochondrial serine transfer RNA lacking the "dihydrouridine" loop and stem. Nucl Acids Res 8:5213–5522

Deutscher MP (1990) Ribonucleases, tRNA nucleotidyltransferase, and the 3′ processing of tRNA. Prog Nucl Acid Res Mol Biol 39:209–240

Dirheimer G, Keith G, Dumas P, Westhof E (1995) Primary, secondary and tertiary structures of tRNAs. In: Soll D, RajBhandary UL (eds) tRNA: structure, biosynthesis, and function. ASM press, Washington DC, pp 93–126

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) Touchdown PCR to circumvent spurious priming during gene amplification. Nucl Acids Res 19:4008

Duchene AM, Pujol C, Mare′chal-Drouard L (2009) Import of tRNAs and aminoacyl-tRNA synthetases into mitochondria. Curr Genet 55:1–18

Esseiva AC, Marechal-Drouard L, Cosset A, Schneider A (2004) The t-stem determines the cytosolic or mitochondrial localization of Trypanosomal tRNAsMet. Mol Biol Cell 15:2750–2757

Frohman MA (1990) RACE: rapid amplification of cDNA ends. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols, a guide to methods and applications. Academic Press Inc, California, pp 28–38

Fukami H, Knowlton N (2005) Analysis of complete mitochondrial DNA sequences of three members of the coral species complex (Cnidaria, Anthozoa, Scleractinia). Coral Reefs 24:410–417

Gissi C, Iannelli F, Pesole G (2008) Evolution of the mitochondrial genome of metazoa as exemplified by comparison of congeneric species. Heredity 101:301–320

Haen KM, Pett W, Lavrov DV (2010) Parallel loss of nuclear-encoded mitochondrial aminoacyl-tRNA synthetases and mtDNA-encoded tRNAs in Cnidaria. Mol Biol Evol 27:2216–2219

Hancock K, Hajduk SL (1990) The mitochondrial tRNAs of *Trypanosoma brucei* are nuclear-encoded. J Biol Chem 265:19208–19215

Helfenbein KG, Fourcade HM, Vanjani RG, Boore JL (2004) The mitochondrial genome of *Paraspadella gotoi* is highly reduced and reveals that chaetognaths are a sister group to protostomes. Proc Natl Acad Sci USA 101:10639–10643

Helm MH, Brule F, Degoul F, Cepanec C, Leroux J-P, Geige R, Florentz C (1998) The presence of modified nucleotides is required for cloverleaf folding in a human mitochondrial tRNA. Nucl Acids Res 26:1636–1643

Juhling F, Morl M, Hartmann RK, Sprinzl M, Stadler PF, Putz J (2009) Compilation of tRNA sequences and tRNA genes. Nucleic Acids Res 37(1):D159–D162

Kayal E, Lavrov DV (2008) The mitochondrial genome of *Hydra oligactis* (Cnidaria, Hydrozoa) sheds new light on animal mtDNA evolution and cnidarian phylogeny. Gene 410:177–186

Lavrov DV (2007) Key transitions in animal evolution: a mitochondrial DNA perspective. Integr Comp Biol 47:734–774

Lavrov DV (2011) Key transitions in animal evolution: a mitochondrial DNA perspective. In: Desalle R, Schierwater B (eds) Key transitions in animal evolution. Science publishers & CRS Press, Enfield, pp 35–54

Lill R, Lepier A, Schwagele F, Sprinzl M, Vogt H, Wintermeyer W (1988) Specific recognition of the 3′-terminal adenosine of $tRNA^{Phe}$ in the exit site of *E. coli* ribosomes. J Mol Biol 203:699–705

Limbach PA, Crain PF, McCloskey JA (1994) Summary: the modified nucleosides of RNA. Nucl Acids Res 22:2183–2196

Liu M, Horowitz J (1994) Functional transfer RNAs with modifications in the 3′-CCA end: differential effects on aminoacylation and polypeptide synthesis. Proc Natl Acad Sci USA 91:10389–10393

Marechal-Drouard L, Guillemaut P, Cosset A, Arbogast M, Weber F, Weil JH, Dietrich A (1990) Transfer RNAs of potato (*Solanum tuberosum*) mitochondria have different genetic origins. Nucl Acids Res 18:3689–3696

Marechal-Drouard L, Weil JH, Guillemaut P (1988) Import of several tRNAs from the cytoplasm into the mitochondria in bean *Phaseolus vulgaris*. Nucl Acids Res 16:4777–4788

Marck C, Grosjean H (2002) tRNomics: analysis of tRNA genes from 50 genomes of Eukarya, Archaea, and Bacteria reveals anticodon-sparing strategies and domain-specific features. RNA 8:1189–1232

Martin NC (1995) Organellar tRNAs: biosynthesis and function. In: Soll D, RajBhandary UL (eds) tRNA: structure, biosynthesis, and function. ASM press, Washington DC, pp 127–140

Medina M, Collins AG, Takaoka TL, Kuehl JV, Boore JL (2006) Naked corals: skeleton loss in Scleractinia. Proc Natl Acad Sci 103:9096–9100

Montiel R, Lucena MA, Medeiros J, Simoes N (2006) The complete mitochondrial genome of the entomopathogenic nematode *Steinernema carpocapsae*: insights into nematode mitochondrial DNA evolution and phylogeny. J Mol Evol 62:211–225

Nagaike T, Suzuki T, Tomari Y, Takemoto-Hori C, Negayama F, Watanabe K, Ueda T (2001) Identification and characterization of mammalian mitochondrial tRNA nucleotidyltransferases. J Biol Chem 276:40041–40049

Okimoto R, Macfarlane JL, Wolstenholme DR (1990) A set of tRNAs that lack either the TψC arm or the dihydrouridine arm: towards a minimal tRNA adaptor. EMBO J 9:3405–3411

Okimoto R, Macfarlane JL, Clary DO, Wolstenholme DR (1992) The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. Genetics 130:471–498

Pont-Kingdon GA, Beagley CT, Okimoto R, Wolstenholme DR (1994) Mitochondrial DNA of the sea anemone, *Metridium senile* (Cnidaria): prokaryote-like gene for $tRNA^{f\text{-}Met}$ and small subunit ribosomal RNA, and standard genetic code specificities for AGR and ATA codons. J Mol Evol 39:387–399

Pont-Kingdon GA, Vassort CG, Warrior R, Okimoto R, Beagley CT, Wolstenholme DR (2000) Mitochondrial DNA of *Hydra attenuata* (Cnidaria): a sequence that includes an end of one linear molecule and the genes for l-rRNA, $tRNA^{f\text{-}Met}$, $tRNA^{Trp}$, COII, and ATPase8. J Mol Evol 51:404–415

Pont-Kingdon GA, Okada NA, Macfarlane JL, Beagley CT, Watkins-Sims CD, Cavalier-Smith T, Clark-Walker GD, Wolstenholme DR (1998) Mitochondrial DNA of the coral *Sarcophyton glaucum* contains a gene for a homologue of bacterial MutS: a possible case of gene transfer from the nucleus to the mitochondrion. J Mol Evol 46:419–443

RajBhandary UL, Chow CM (1995) Initiator tRNAs and the initiation of protein synthesis. In: Soll D, RajBhandary UL (eds) tRNA: structure, biosynthesis, and function. ASM press, Washington DC, pp 511–528

Randerath E, Hari P, Agrawal HP, Randerath K (1981) Rat liver mitochondrial lysine tRNA (anticodon U*UU) contains a rudimentary D-arm and 2 hypermodified nucleotides in its anticodon loop. Biochem Biophys Res Commun 103:739–744

Rickwood D, Wilson MT, Darley-Usmar VM (1987) Isolation and characteristics of intact mitochondria. In: Darley-Usmar VM, Rickwood D, Wilson MT (eds) Mitochondria: a practical approach. IRL Press, Washington DC, pp 1–16

Roe BA, Wong JFH, Chen EY, Armstrong PW, Stankeiwicz A, Ma D-P, McDonough J (1982) Mammalian mitochondrial tRNAs: a modified nucleotide 3′ to the anticodon may modulate their codon response. In: Slonimsky P, Borst P, Attardi G (eds) Mitochondrial genes. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp 45–49

Samaha RR, Green R, Noller HF (1995) A base pair between tRNA and 23S rRNA in the peptidyl transferase centre of the ribosome. Nature (London) 377:309–314

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schneider A (2011) Mitochondrial tRNA import and its consequences for mitochondrial translation. Ann Rev Biochem 80:1033–1053

Simpson AM, Suyama Y, Dewes H, Campbell DA, Simpson L (1989) Kinetoplastid mitochondria contain functional tRNAs which are encoded in nuclear DNA and also contain small minicircle and maxicircle transcripts of unknown function. Nucl Acids Res 17:5427–5445

Shao Z, Graf S, Chaga OY, Lavrov DV (2006) Mitochondrial genome of the moon jelly *Aurelia aurita* (Cnidaria, Scyphozoa): a linear DNA molecule encoding a putative DNA-dependant DNA polymerase. Gene 381:92–101

Sprinzl M, Horn C, Brown M, Ioudovitch A, Teinberg S (2005) Compilation of tRNA sequences and sequences of tRNA genes. Nucl Acids Res 26:148–153

Tamura K (1994) The role of the CCA sequence of tRNA in the peptidyl-transfer reaction. FEBS Lett 353:173–176

Tamura K, Nameki N, Hasegawa T, Shimizu M, Himeno H (1994) Role of the CCA terminal sequence of $tRNA^{Val}$ in aminoacylation with valyl-tRNA synthetase. J Biol Chem 269:22173–22177

Tarassov I, Komenski P, Kolesnikova O, Martin RP, Krasheninnikov IA, Entelis N (2007) Import of nuclear DNA-encoded RNAs into mitochondria and mitochondrial translation. Cell Cycle 6(2473):2477

Tomita K, Weiner AM (2001) Collaboration between CC- and A-adding enzymes to build and repair the 3′-terminal CCA of tRNA in *Aquifex aeolicus*. Science 294:1334–1336

van Oppen MJ, Catmull J, McDonald BJ, Hislop NR, Hagerman PJ, Miller DJ (2002) The mitochondrial genome of *Acropora tenuis* (Cnidaria; Scleractinia) contains a large group I intron and a candidate control region. J Mol Evol 55:1–13

Watanabe K, Yokobori S (2011) tRNA modification and genetic code variations in animal mitochondria. J Nucl Acids 623095

Wolstenholme DR (1992) Animal mitochondrial DNA: structure and evolution. In: Wolstenholme DR, Jeon KW (eds) Mitochondria genomes. International review of cytology, vol 141. Academic Press, New York, pp 173–216

Wolstenholme DR, Macfarlane JL, Okimoto R, Clary DO, Wahleithner JA (1987) Bizarre tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms. Proc Natl Acad Sci USA 84:1324–1328

Wolstenholme DR, Okimoto R, Macfarlane JL (1994) Nucleotide correlations that suggest tertiary interactions in the TV-replacement loop-containing mitochondrial tRNAs of the nematodes, *Caenorhabditis elegans* and *Ascaris suum*. Nucl Acids Res 22:4300–4306

Wolstenholme DR, Fauron CM-R (1995) Mitochondrial genome organization. In Levings III CS, Kluwer VIK (eds) Advances in cellular and molecular biology of plants. Molecular biology of the mitochondria, vol 3. Academic Publishers, the Netherlands, pp 1–59