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

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Manuscript received September 21, 1998
Accepted for publication March 5, 1999

ABSTRACT

A 2500-nucleotide pair (ntp) sequence of F-type mitochondrial (mt) DNA of the Pacific Rim mussel *Mytilus californianus* (class Bivalvia, phylum Mollusca) that contains two complete (ND2 and ND3) and two partial (COI and COIII) protein genes and nine tRNA genes is presented. Seven of the encoded tRNAs (Ala, Arg, His, Met(AUA), Pro, Ser(UCN), and Trp) have the potential to fold into the orthodox four-armed tRNA secondary structure, while two [tRNA^{Ser}(AGN) and a second tRNA^{Ser}(UCN)] will fold only into tRNAs with a dihydrouridine (DHU) arm-replacement loop. Comparison of these mt-tRNA gene sequences with previously published, corresponding *M. edulis* F-type mtDNA indicates that similarity between the four-armed tRNA^{Ser}(UCN) genes is only 63.8% compared with an average of 92.1% (range 86.2–98.5%) for the remaining eight tRNA genes. Northern blot analysis indicated that mature tRNAs encoded by the DHU arm-replacement loop-containing tRNA^{Ser}(UCN), tRNA^{Ser}(AGN), tRNA^{Met}(AUA), tRNA^{Trp}, and tRNA^{Pro} genes occur in *M. californianus* mitochondria, strengthening the view that all of these genes are functional. However, Northern blot and 5′ RACE (rapid amplification of cDNA ends) analyses indicated that the four-armed tRNA^{Ser}(UCN) gene is transcribed into a stable RNA that includes the downstream COI sequence and is not processed into a mature tRNA. On the basis of these observations the *M. californianus* and *M. edulis* four-armed tRNA^{Ser}(UCN) sequences are interpreted as pseudo-tRNA^{Ser}(UCN) genes.

THE mitochondrial (mt) genomes of almost all multicellular animals (metazoa) comprise a single circular molecule of 14–42 kb (Wolstenholme 1992a; Wolstenholme and Fauron 1995). The mtDNA molecules of ~75 metazoans have been completely sequenced. Approximately two-thirds of these are from chordates (Krettek *et al.* 1995; Arnason *et al.* 1996; Zardoya and Meyer 1997) but the remainder represent a variety of invertebrate phyla, including Cnidaria, Nematoda, Arthropoda, Mollusca, Annelida, and Echinodermata (see, for example, Clary and Wolstenholme 1985; Jacobs *et al.* 1988; Cantatore *et al.* 1989; Garey and Wolstenholme 1989; Okimoto *et al.* 1991, 1992; Crozier and Crozier 1993; Boore and Brown 1994, 1995; Asakawa *et al.* 1995; Beagley *et al.* 1995, 1998; Hatzoglou *et al.* 1995; Terrett *et al.* 1996).

Most metazoan mtDNAs contain the same set of genes for 13 energy-pathway proteins: cytochrome b (Cyt b); subunits I–III of cytochrome *c* oxidase (COI–COIII); subunits 6 and 8 of F_O ATP synthetase (ATPase6 and ATPase8); subunits 1–6 and 4L of the respiratory chain NADH dehydrogenase (ND1-ND6 and ND4L); and two RNAs that are homologous to the 16S and 23S ribosomal RNAs (rRNAs) of *Escherichia coli* (but smaller and usually referred to as s-rRNA and l-rRNA) and 22 transfer RNAs (tRNAs). The arrangement of genes within metazoan mtDNAs is very compact. Differences in gene arrangement occur among metazoan mtDNAs, and the extent of these differences appears to be a function of evolutionary distances (Wolstenholme 1992a; Boore and Brown 1994, 1995).

Metazoan mt-genomes are characterized by a number of unusual features. These include genetic code modifications, the use of unorthodox translation initiation codons, post-transcriptional generation of translation termination codons, translation by only 22 tRNAs that are mtDNA encoded and of diverse size and structural form, and replication by a unique asymmetrical mode (Clayton 1992; Wolstenholme 1992a,b; Wolstenholme and Fauron 1995).

Hoffmann *et al.* (1992) determined nucleotide sequences that collectively represent ~80% of the ~17.1-kb mtDNA molecule of the blue mussel *Mytilus edulis* (phylum Mollusca, class Bivalvia). Because these sequences contained the terminal 5′ and 3′ ends of all protein and rRNA genes and all sequences between these genes including tRNA genes, they provided sufficient information to construct a complete gene map.

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The *M. edulis* mtDNA molecule was found to contain the gene complement common to fully mapped metazoan mtDNAs with two exceptions. The first is that there are two genes for methionine-specifying tRNAs: one with a CAU anticodon ($tRNA^{f\text{-}Met}$), as has been reported for all other metazoan mtDNAs so far examined, and a second with a UAU anticodon that might be expected to specifically recognize AUA codons, and is so far unique among metazoan mtDNAs. The second exception to gene content is that a gene for ATPase8 was not identified, a situation previously known among Metazoa only for nematode mtDNAs (Wolstenholme *et al.* 1987; Okimoto *et al.* 1991, 1992). Relative to the corresponding genes of *Drosophila yakuba* and mouse, considerable extensions were postulated at the 5′ ends of the COI and ND1 genes, and at the 3′ ends of the COI and COIII genes of *M. edulis*. The COIII gene 3′ extension was of particular interest as the overall size of this gene and the C-terminal proximal amino acid sequence of the protein it encodes have been highly conserved in all organisms previously examined. Gene arrangement in the mtDNA molecule of *M. edulis* was found to be greatly different from gene arrangement in other sequenced metazoan mtDNAs, including those of the more recently sequenced polyplacophoran mollusk *Katherina tunicata* and the gastropod mollusks *Albinaria coerulea* and *Cepaea nemoralis* (Boore and Brown 1994; Hatzoglou *et al.* 1995; Terrett *et al.* 1996).

Subsequent to the report of Hoffmann *et al.* (1992) it was demonstrated that in *M. edulis*, the closely related *M. trossulus* and *M. galoprovincialis*, and the more distantly related *M. californianus*, there are two highly distinct mtDNA sequence types that are gender limited in occurrence (Fisher and Skibinski 1990; Zouros *et al.* 1992, 1994a,b; Skibinski *et al.* 1994a,b; Beagley *et al.* 1997). One mtDNA type (F) is found in both females and males, but the second type (M) is found only in males. The mtDNA molecule sequenced by Hoffmann *et al.* (1992) has been identified as the F-type.

In this article we report the sequence of a 2500-nucleotide pair (ntp) segment of the F-type mtDNA molecule of *M. californianus*. From comparison of this latter sequence with the corresponding *M. edulis* sequence, and data obtained from Northern blot analysis, we show that Mytilus mtDNA molecules include a gene that encodes a functional dihydrouridine (DHU) arm-replacement loop-containing $tRNA^{Ser}$ (UCN), not identified by Hoffmann *et al.* (1992). Further, we demonstrate that the sequence interpreted by Hoffmann *et al.* (1992) as the Mytilus mt-$tRNA^{Ser}$(UCN) gene is likely a pseudogene, the transcript of which remains linked to the transcript of the COI gene. Our data also confirm that the Mytilus mt-$tRNA^{Met}$(AUA) gene encodes a functional tRNA, and provide confirmatory support for the conclusion that the Mytilus cytochrome *c* oxidase subunit III protein (COIII) has a unique, functional C-terminal extension.

MATERIALS AND METHODS

Animals and mitochondrial nucleic acid isolations: Specimens of *M. californianus* were collected from Monterey Bay by Michael O. Morris, Sea Life Supply, Sand City, CA. Mitochondria were isolated from mechanically disrupted adductor muscles of 10 *M. californianus* and mtDNA was prepared by methods previously described (Wolstenholme and Fauron 1976; Pont-Kingdon *et al.* 1994). For the preparation of RNA, total soft tissue was removed from 20 live *M. californianus*, chilled on ice, immersed in 5 volumes of 50 mm Tris-HCl (pH 8.0), 300 mm mannitol, 3 mm EDTA, 0.1% bovine serum albumin, 1 mm β-mercaptoethanol, 4 mm l-cysteine, and 0.1% polyvinylpyrrolidone, and mechanically disrupted in a Waring blender. The product was centrifuged for 15 min at 4,000 × *g*. From the resulting supernatant a crude mitochondrial pellet was obtained by centifugation for 20 min at 10,000 × *g*, and RNA was isolated from this pellet with a REX Total RNA extraction kit (Amersham Life Science, Piscataway, NJ), using a mitochondrial weight equivalent to that recommended by the supplier for total animal tissue. Finally, the RNA was ethanol (3 volumes 95%) precipitated, resuspended in diethylpyrocarbonate-treated water, and either used directly or stored at −20°.

Oligonucleotide probes: Oligonucleotides were synthesized in an Applied Biosystem (Foster City, CA) Synthesizer 380B and used without further purification. Oligonucleotides complementary to the sense strand of various presumptive tRNA genes and the ψ$tRNA^{Ser}$(UCN) gene (oligonucleotide F5) that were used in Northern blot experiments are shown in Figure 2. The sequence of an oligonucleotide complementary to *E. coli* $tRNA^{Tyr}$ and used as a size marker in one Northern experiment was 5′GGAAGGATTCGAACCTTCGAA. These oligonucleotides were 5′-end-labeled using T4 polynucleotide kinase (BRL) and [γ-^{32}P]ATP. The gene locations of all other oligonucleotides (F1–F4) used in Northern blot experiments and/or as primers in PCR amplifications are shown in Figure 1B, and following are their nucleotide sequences together with their coordinates in the determined sequence (Figure 1A). Reverse-order numbers correspond to antisense sequences. Nonencoded restriction site-containing sequences are bracketed. F1: 1–30, 5′ [TGGAATTC]TGGTACTGACTTTTGTAGATG; F2: 3195–3178 (not included in Figure 1A), 5′ [AGGAATT]CCA GAACAATGCATAATTACC; F3: 2012–1992, 5′ TAAATA GAAACCCTCTTGAGG; and F4: 2402–2383, 5′ CTACTTCAC CGTAACCGCCTC. Following are the nucleotide sequences of degenerate primers COI-11D and COI-12D (Figure 1B), which are based on highly conserved sequences in various metazoan COI genes. The coordinates shown are those of the *Metridium senile* intron-containing COI gene complex (Beagley *et al.* 1996). Y = C or T, R = A or G, N = C, T, A, or G. Nonencoded, restriction site-containing sequences are bracketed. COI-11D: 712–731, 5′ [CCGGATCC]TTYTGRTTYTTYG GNCAYCC; COI-12D: 1982–1964 (equivalent to ntp 1051–1070 of the uninterrupted COI gene), 5′ [CCGGATCC]AC NACRTARTANGTRTCRTG.

PCR amplification of *M. californianus* mtDNA: A 435-ntp segment of the *M. californianus* COI gene was PCR amplified using the degenerate primer pair COI-11D and COI-12D, and then sequenced. An oligonucleotide (F2) based on this COI sequence, which included an added 5′ end *Eco*RI site, together with an oligonucleotide (F1) corresponding to a segment of the *M. edulis* COIII gene, and also including a 5′ end *Eco*RI site, were used to PCR amplify a 3.2-kb segment of *M. californianus* mtDNA. This 3.2-kb sequence was cloned in the two reverse orientations into bacteriophage M13mp19 DNA, and amplified in *E. coli* JM101. The two reversely oriented, cloned sequences were designated MC412 and MC413. Except as described below, details regarding PCR amplification, electro-

phoresis, cloning, and purification of single-strand M13 DNA are given or referred to in Wahleithner and Wolstenholme (1987) and Beagley *et al.* (1996, 1998).

DNA sequencing and sequence analysis: DNA sequences were obtained by the extension-dideoxy-termination procedure (Sanger *et al.* 1977) from nested sets of deletion clones (Dale *et al.* 1985) of each of the two M13mp19 *M. californianus* mtDNA clones MC412 and MC413 that represent both strands of the 2500-ntp segment shown in Figure 1A, all of the remaining 684 nt of one strand (MC412), and 393 nt of the complementary strand (MC413). Sequences were analyzed using Lasergene software from DNASTAR (Madison, WI). The 2500-ntp *M. californianus* mtDNA sequence has been submitted to GenBank under the accession no. AF090831.

Northern blotting: For determination of approximate relative sizes of RNAs containing transcripts of some tRNA genes and the ψtRNA^{Ser}(UCN) gene, *M. californianus* whole mtRNA (3 μg/lane) was electrophoresed in a 2.2 m formaldehyde-containing 3% agarose gel, transferred to a nitrocellulose filter, and hydridized with ^{32}P-labeled gene sequence-specific oligonucleotides. A similar procedure was used to determine and compare the sizes of RNAs containing transcripts of the ND3, COI, and ψtRNA^{Ser}(UCN) genes, except that electrophoresis was carried out in a 2.2 m formaldehyde-containing 1% agarose gel.

For precise determination of tRNA sizes, *M. californianus* mtRNA (10 μg/lane) was electrophoresed in polyacrylamide gels, electroblotted, and probed with gene sequence-specific oligonucleotides as described in Okimoto and Wolstenholme (1990), with the following exceptions: the 15% polyacrylamide gel (containing 8 m urea) was 17 cm wide by 15 cm long; electrophoresis was continued until the cyanol dye ran completely off the gel; the blots were washed twice at 25° in 2× SSC, 0.1% SDS for 10 min, followed by two washes at hybridization temperature (37° rather than 35°) in 0.1× SSC, 0.1% SDS for 30 min each.

Location of the 5′ end of the COI transcript: 5′ RACE (rapid amplification of cDNA ends) analysis (Frohman 1990) was carried out to locate the 5′ end of COI transcript-containing RNA molecules as follows: *M. californianus* whole mtRNA (10 μg) was reverse transcribed in a 100-μl reaction using 1.5 μm random hexamers (BRL) and 1000 units of Moloney-MLV reverse transcriptase under conditions recommended by the supplier (Gibco BRL, Gaithersburg, MD). Incubation was for 10 min at 25° followed by 30 min at 42°. Following extraction of the incubation product with phenol, then chloroform, cDNAs were purified using a Centricon 100 microconcentrator (Amicon, Beverly, MA). Deoxyadenosine monophosphates (dAs) were added to the 3′ ends of cDNAs by incubation in a 50-μl reaction containing cDNAs, 2.0 μl of 10 mm dATP, and 34 units of terminal deoxynucleotidyl transferase (Amersham) under the supplier's recommended reaction conditions for 5 min at 37°. Following a further phenol/chloroform extraction and Centricon purification, a second-strand synthesis reaction was carried out under the reverse transcription conditions but using as primer 20 pm of a *Bam*HI, *Bgl*II, and *Pst*I site-containing adaptor oligonucleotide (TB17-1: 5′ CCAGATCGGATCCTGACGTTTTTTTTTTTTTTT; Pont-Kingdon *et al.* 1994). The product was phenol/chloroform extracted, Centricon purified, and taken up in 50 μl of Tris-EDTA (TE) to provide a 5′ RACE pool. Amplification of the double-stranded cDNA that included the 5′ end region of the COI transcript was accomplished in a 100-μl PCR (in a DNA thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) using 5 μl of the RACE pool, 25 pm of a *Bam*HI, *Bgl*II, *Pst*I site-containing primer TB17-2 (5′ CCAGATCTGGATCCTGCAG), 25 pm of oligonucleotide F4 [which is complementary to a nucleotide sequence within the 5′ end region of the *M. californianus* COI gene (Figure 2)], and an annealing temperature of 58°. The PCR product was digested with *Bam*HI, inserted into *Bam*HI-cleaved M13mp19, and sequenced.

RESULTS AND DISCUSSION

Complete 2500-ntp sequences were obtained from two reversely oriented inserts of a PCR-generated 3.2-kb *M. californianus* mtDNA segment, cloned in M13mp19. As the two 2500-ntp sequences obtained were identical, we concluded that they both originated from the same mtDNA type. That this was the F-type was determined by comparison of a portion of the COI gene sequence with the equivalent COI F-type and COI M-type sequences identified by Beagley *et al.* (1997).

The entire, continuous 2500-ntp sequence of *M. californianus* F-type mtDNA is given in Figure 1A, and is compared with two sequences of *M. edulis* F-type mtDNA reported by Hoffmann *et al.* (1992). We interpret the *M. californianus* sequence to contain the following genes in the order given: COIII (3′ end region), tRNA^{Ser}(UCN), tRNA^{Met}(AUA), ND2, tRNA^{Arg}, tRNA^{Trp}, tRNA^{Ala}, tRNA^{Ser}(AGN), tRNA^{His}, tRNA^{Pro}, ND3, ψtRNA^{Ser}(UCN); (see below), and COI (5′ end region). The two *M. edulis* sequences, collectively, have the same gene content, but the sequence of a central region of the ND2 gene was not determined (Figure 1B).

Protein genes: Hoffmann *et al.* (1992) found that the *M. edulis* COI gene-containing open reading frame extended upstream beyond the codon corresponding to the translation initiation codons of the mouse and *D. yakuba* COI genes, for 21 and 22 codons, respectively. They suggested that, based on available information, a number of different triplets might act as the *M. edulis* COI translation initiation codon, and tentatively assigned this function to the ATA codon located 8 and 9 codons, respectively, upstream from the mouse and *D. yakuba* COI initiation codons, and at the same relative location as the *Caenorhabditis elegans* and *Ascaris suum* COI initiation codons. However, neither this ATA codon nor the ATT and ATA codons at the beginning of the *M. edulis* COI-gene containing open reading frame are conserved in the *M. californianus* sequence (Figure 1A). Therefore, from considerations of nucleotide sequence and predicted amino acid sequence conservation, it seems most likely that translation initiation of the COI gene occurs at the ATG codon in the open reading frame of both Mytilus species, located 17 and 18 codons upstream from the mouse and *D. yakuba* translation initiation codons (Figure 1A).

Data from nucleotide and amino acid sequence comparisons of the complete *M. californianus* and *M. edulis* ND3 genes, corresponding segments of the complete *M. californianus* ND2 gene and the two sequences of the *M. edulis* ND2 gene, and the portions of the *M. californianus* and *M. edulis* COIII genes that extend 3′ relative to other metazoan COIII genes are given in

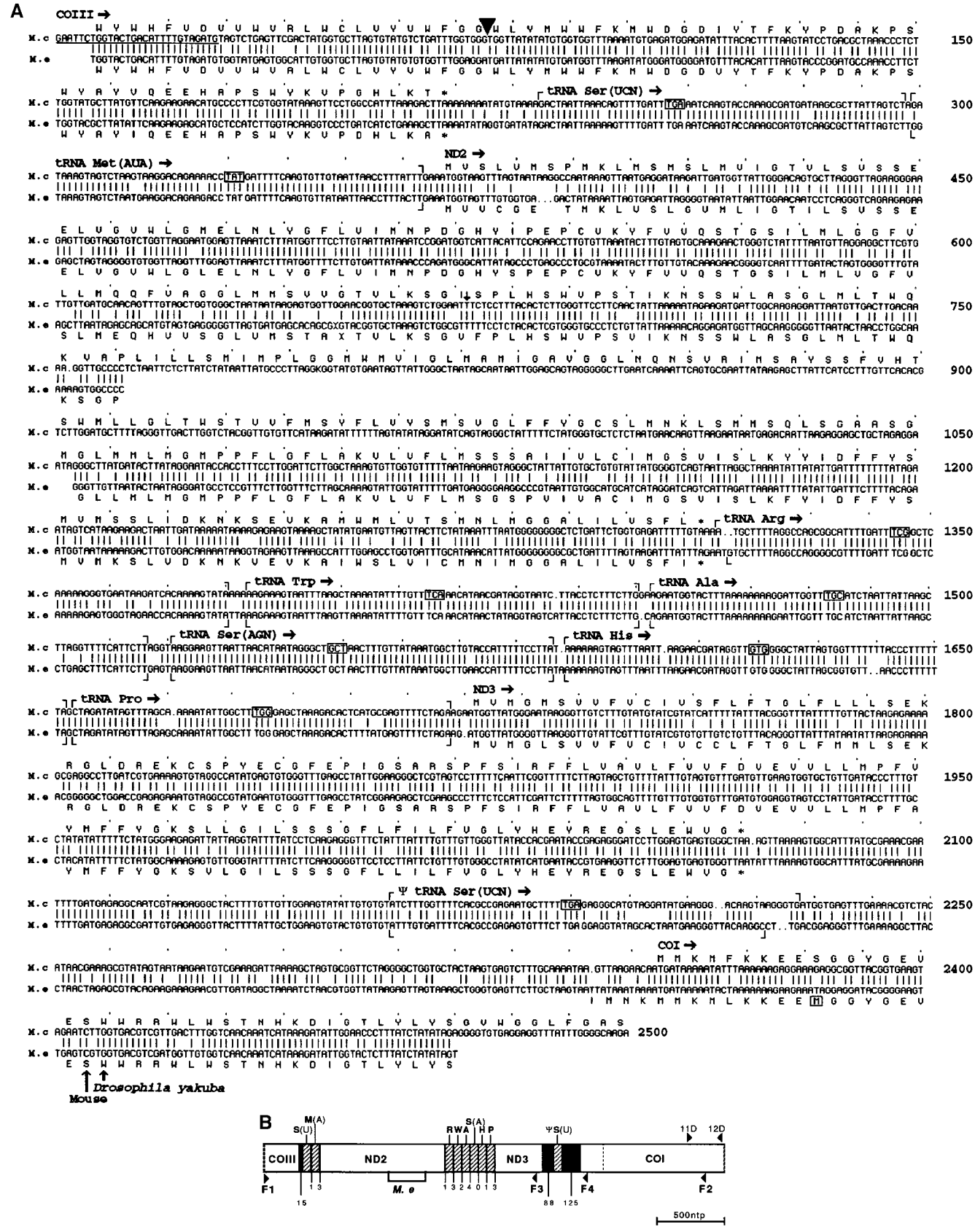

Figure 1.—(A) Comparison of a continuous 2500-ntp sequence of the F-type mtDNA molecule of *M. californianus* (*M.c*) and two corresponding, partial F-type mtDNA sequences of *M. edulis* (*M.e*) taken from Hoffmann *et al.* (1992) (GenBank accession nos. M83760 and M83761). The numbering to the right is shown only for the *M. californianus* sequence. All of the partial and complete protein genes shown (COI and COIII, cytochrome *c* oxidase subunits I and III; ND2 and ND3, NADH dehydrogenase

TABLE 1

Nucleotide and amino acid sequence comparisons concerning the complete ND3 gene and partial ND2 and COIII genes contained in *M. californianus* and *M. edulis* F-type mtDNAs

Gene	No. of nucleotides compared[a]	Nucleotide sequence similarity (%)	Amino acid sequence similarity (%)	Silent substitutions (%)	Transition:transversion ratio	
					Silent positions	All positions
ND3	348	80.2	94.0	82.6	2.4:1	1.9:1
ND2	642	73.2	80.7	71.5	1.8:1	1.6:1
COIII[b]	144	79.9	91.7	86.2	5.3:1	6.3:1

Calculated from data in Figure 1. Data for *M. edulis* are from Hoffmann *et al.* (1992).
[a] Excluding termination codons.
[b] Extension only.

Table 1. For the ND3 and ND2 genes, nucleotide and amino acid sequence similarities are 80.2 and 73.2%, and 94 and 80.7%, respectively. The corresponding values for comparisons of the *M. californianus* and *M. edulis* COIII 3′ extensions are intermediate to those obtained for the ND3 and ND2 genes: 79.9 and 91.7%, respectively. Between the ND3 and ND2 genes of the two species, silent substitutions are in excess of replacement substitutions, and in silent positions, transitions exceed transversions, as has been reported for mt-protein genes of closely related species of other invertebrates and vertebrates (Brown *et al.* 1982; Brown and Simpson 1982; Desalle *et al.* 1987). Between the *M. californianus* and *M. edulis* COIII extension sequences the proportion of substitutions that are silent, and the proportion of silent substitutions that are transitions are both greater than found for the ND3 and ND2 gene comparisons (Table 1). Taken together, similarities between patterns of nucleotide substitution among the COIII gene extensions and the ND3 and ND2 genes suggest that the COIII extension sequences have been subjected to selective pressures similar to those that have operated on the ND3 and ND2 gene sequences. Therefore, these data are consistent with the view (Hoffmann *et al.* 1992) that the extra 46 codons of the *M. californianus* and *M. edulis* COIII genes encode a unique functional segment of the Mytilus COIII protein.

Transfer RNA genes: Comparisons of the sequences of the *M. californianus* and *M. edulis* tRNA genes are given in Figure 2 and Table 2. The sequences of eight of the tRNA genes [Ala, Arg, His, Met, Pro, Trp, Ser(AGN), and Ser(UCN)] are highly conserved in regard to primary structure (mean, 92.1%; range, 86.2–98.5%; Table 2) and their potential to fold into secondary structures with features characteristic of mt-tRNAs (Figure 2). Compared with the *M. edulis* sequences, the *M. californianus* sequences have an average of only 4.5 nucleotide substitutions (range 1–9), 0.4 nucleotide deletions

subunits 2 and 3), the eight tRNA genes [Ser(UCN), Met (AUA), Arg, Trp, Ala, Ser(AGN), His, and Pro], and the sequence interpreted as a pseudo-tRNA^{Ser}(UCN) gene [ψSer(UCN)] are transcribed in the same direction, from left to right. Nucleotides 1–30 of the *M. californianus* sequence are one of a pair of PCR primers used to obtain the sequence: an *Eco*RI site followed by 24 nt corresponding to the *M. edulis* sequence. The sequence of the *M. edulis* ND2 gene corresponding to ntp 762–1053 of the *M. californianus* sequence, and the sequence of the *M. edulis* COI gene beyond that shown have not been determined. The anticodon of each tRNA gene is boxed. The inverted large arrowhead above the *M. californianus* COIII sequence indicates the position at which the COIII sequences of other mtDNAs terminate. A period indicates the absence in one nucleotide sequence of a nucleotide that occurs in the other nucleotide sequence. Vertical lines indicate similarities between the *M. californianus* and *M. edulis* nucleotide sequences. The predicted amino acid sequences of the protein genes are shown above the *M. californianus* sequence and below the *M. edulis* sequence. Termination codons are indicated by an asterisk. Below the last line of the *M. edulis* sequence are indicated amino acids corresponding in position to amino acids interpreted as the initial amino acids of predicted mouse and *D. yakuba* COI proteins. The boxed M (methionine) near the 5′ proximal end of the COI gene is the amino acid tentatively interpreted as the first in the polypeptide product of this gene in *M. edulis* by Hoffmann *et al.* (1992). The small arrow indicates a nucleotide in the *M. californianus* sequence that (as shown) was T in each of two clones of one strand, but a C in each of two clones of the complementary strand. Modifications of the standard genetic code used in translation of the Mytilus mtDNA sequences are AGA and AGG = serine; TGA = tryptophan; ATA = methionine. An unspecified nucleotide and amino acid in one of the *M. edulis* sequences is indicated by X. (B) Gene map of the sequenced segment of *M. californianus* mtDNA. The arrowheads above and below the map show the locations and directions of the oligonucleotide primers (F1–F4) and degenerate primers in the COI gene (11D and 12D) used for PCR amplifications. The sequences of all of these oligonucleotides are given in materials and methods. The dotted line near the 5′ end of the COI gene corresponds to the 3′ end of the sequenced segment shown in A. Crosshatched regions are tRNA genes identified above the map using the one-letter amino acid code. Serine and methionine tRNA genes are also identified by the codon family that the tRNA recognizes: S(A), Ser(AGN); S(U), Serine (UCN); M(A), Met(AUA); ψS(U), ψSer(UCN). The numbers of nucleotides in sequences between identified genes (the largest three in black) are shown beneath the map.

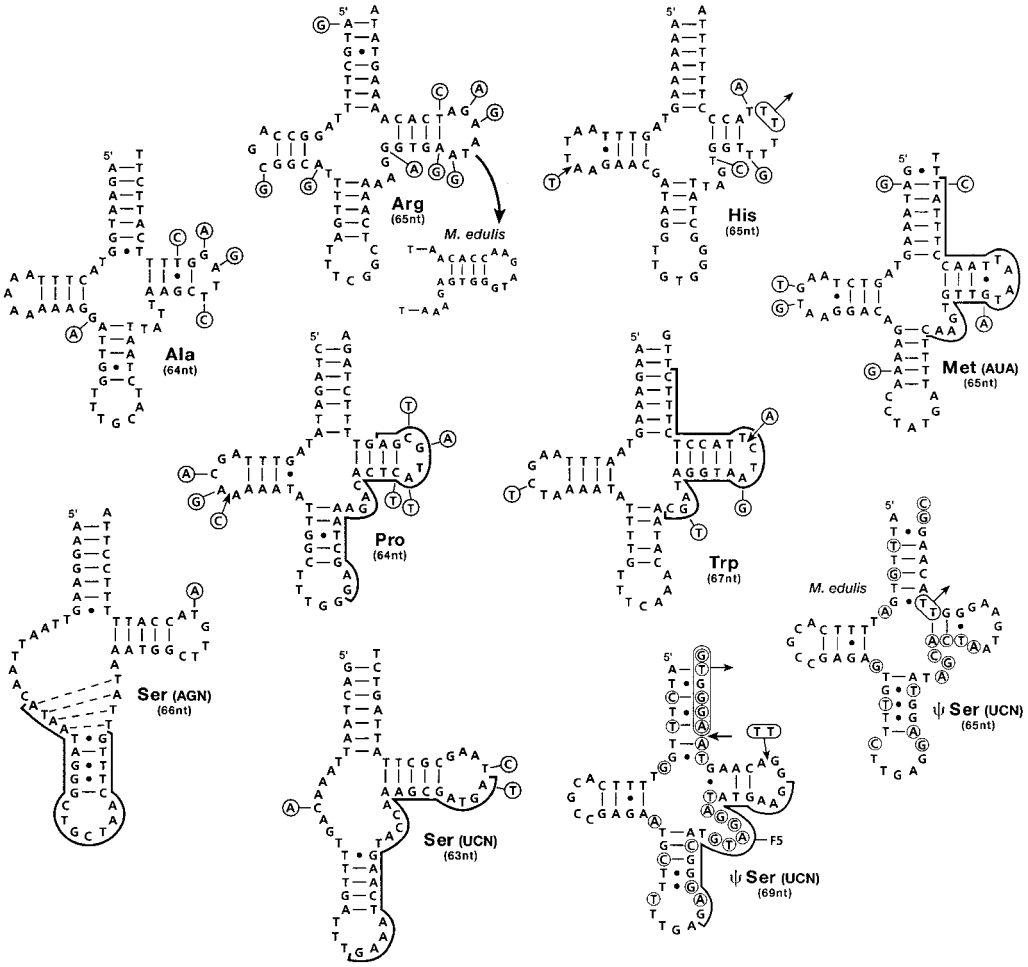

Figure 2.—The eight mt-tRNA genes and the ψtRNA^{Ser}(UCN) gene of *M. californianus* contained in the sequence in Figure 1, and the ψtRNA^{Ser}(UCN) gene of *M. edulis* (Hoffmann *et al.* 1992), shown in the predicted secondary structural forms of the corresponding tRNAs. Each solid line on the outside of some *M. californianus* tRNA genes, and the solid line (F5) on the outside of the *M. californianus* ψtRNA^{Ser} (UCN) gene, identifies a segment to which a complementary oligonucleotide was made and used in hybridization experiments to test for the presence of the various corresponding tRNAs. The identity (derived from the anticodon) and size (in nucleotides, nt) of each tRNA gene are shown. In the DHU arm-replacement loop-containing tRNA^{Ser} (AGN) gene, possible secondary interactions between nucleotides in the DHU arm-replacement loop and the variable loop are shown by dashed lines. Reconstruction of the TψC arm of the *M. edulis* tRNA^{Arg} gene relative to that given by Hoffmann *et al.* (1992), based on comparisons of the *M. californianus* and *M. edulis* tRNA^{Arg} gene sequences, is shown. In each functional (or presumed functional) *M. californianus* tRNA gene, nucleotide substitutions that occur in the corresponding *M. edulis* tRNA gene (Hoffmann *et al.* 1992) are shown circled outside the sequence. Nucleotides in the *M. californianus* sequence that are circled with an arrow pointing out are not found in the *M. edulis* sequence. Arrows attached to circled nucleotides outside the *M. californianus* sequences indicate the locations of nucleotides found in the *M. edulis* tRNA sequences but not in the *M. californianus* sequences. Circled nucleotides in the *M. californianus* ψtRNA^{Ser}(UCN) gene and the *M. edulis* ψtRNA^{Ser}(UCN) gene are those that are substituted in the corresponding sequence of the other species.

(range 0–1), and 0.3 nucleotide insertions (range 0–2; Table 2). These data clearly support the interpretation of each of these sequences as an mt-tRNA gene. The tRNA^{Ser}(UCN) gene, located between the COIII and tRNA^{Met}(AUA) genes, contains an 8 nucleotide (nt)-loop that replaces the DHU arm. A similar secondary structure is predicted for the tRNA^{Ser}(AGN) gene (Figure 2). Although a similarly located sequence [between the COIII and tRNA^{Met}(AUA) genes] in *M. edulis* mtDNA was noted to have stable secondary structure potential, it was not identified as a tRNA^{Ser}(UCN) gene by Hoffmann *et al.* (1992). Rather, they interpreted a sequence located between the ND3 and COI genes as the *M. edulis* mt-tRNA^{Ser}(UCN) gene. This sequence could be folded into a secondary structure that included most of the characteristic features expected for an mt-tRNA (Figure 2). A sequence in the corresponding location in *M. californianus* mtDNA can also be folded into a four-armed mt-tRNA-like structure (Figure 2). However, the sequences that form the TψC arm and the 3′ strand of the aminoacyl stem are not colinear in the mt-tRNA-like structures of the two species. Also, relative to the sequence contained in the *M. californianus* tRNA-like structure, there are 23 nucleotide substitutions in the corresponding *M. edulis* sequence. This degree of divergence contrasts with the highly conserved sequences of the other eight mt-tRNA genes of the two species (mean divergence = 5.1 nucleotides/tRNA; Figure 2, Table 2) and strongly supports the interpretation of this region of the two mt-tRNAs as a pseudogene that we designate ψtRNA^{Ser}(UCN).

Experiments were carried out to test for the presence in *M. californianus* mitochondria of RNAs corresponding to the tRNA^{Ser}(UCN), tRNA^{Ser}(AGN), tRNA^{Trp}, and tRNA^{Pro} genes and the ψtRNA^{Ser}(UCN) sequence. Oligonucleotides complementary to each of these genes and the ψtRNA^{Ser}(UCN) sequence (Figure 2) were synthesized, ^{32}P-labeled, and hybridized to a nitrocellulose blot

TABLE 2

Comparisons of nucleotide sequences in eight tRNA genes and a ψtRNA gene in *M. californianus* F-type mtDNA with the nucleotide sequences of the corresponding tRNA genes of *M. edulis* F-type mtDNA

M. californianus F-type		Compared with *M. edulis* F-type tRNA genes				
		Number of nucleotide changes				
tRNA gene	Total nucleotides	Substitutions	Deletions	Insertions	Total	Percentage identity (divergence)
Ala	64	5	0	0	5	92.2 (7.8)
Arg	65	9	0	0	9	86.2 (13.8)
His	65	3	1	2	6	91.0 (9.0)
Met(AUA)	65	6	0	0	6	90.8 (9.2)
Pro	64	6	1	0	7	89.1 (10.9)
Trp	67	3	1	0	4	94.0 (6.0)
Ser(AGN)	66	1	0	0	1	98.5 (1.5)
Ser(UCN)	63	3	0	0	3	95.2 (4.8)
Mean of eight tRNA genes	64.9	4.5	0.4	0.3	5.1	92.1 (7.9)
ψSer(UCN)[a]	69	23	2	0	25	63.8 (36.2)

Data for *M. edulis* are from Hoffmann *et al.* (1992).

[a] The comparison is for the entire *M. californianus* ψtRNA^{Ser}(UCN) sequence (Figures 1 and 2) and the *M. edulis* sequence corresponding to it, which includes the entire shorter *M. edulis* ψtRNA^{Ser}(UCN) sequence (Figure 2).

of whole mtRNA that had been electrophoresed in a 3% denaturing agarose gel. Examination of an autoradiograph of the product (Figure 3) revealed that the probes for each of the tRNA genes, but not that for the ψtRNA^{Ser}(UCN) sequence, had hybridized to an RNA of an approximate size (70 nt) expected for a tRNA. In contrast, the ψtRNA^{Ser}(UCN) probe hybridized to RNA molecules averaging ~2500 nt, indicating that although the ψtRNA^{Ser}(UCN) sequence is transcribed, it is rarely or never processed into a tRNA-sized molecule.

Two further Northern hybridization experiments were carried out to determine the absolute sizes of the various *M. californianus* mt-tRNAs. Both experiments involved hybridizing ^{32}P-labeled tRNA gene-specific oligonucleotides (Figure 2) to blots of mtRNA electrophoretically separated on 15% acrylamide gels (Okimoto and Wolstenholme 1990). In the first experiment, to provide a size marker, *E. coli* tRNA^{Tyr} (85 nt including the DNA-encoded 3′ end-located CCA) was coelectrophoresed in a separate lane in the gel. Oligonucleotides specific for tRNA^{Trp}, tRNA^{Ser}(UCN), and tRNA^{Met}(AUA) sequences and the *E. coli* tRNA^{Tyr} sequence were hybridized separately to blots of the gel. In the second experiment the above-mentioned three oligonucleotides, together with oligonucleotides complementary to the mt-tRNA^{Ser}(AGN) and mt-tRNA^{Pro} genes (Figure 2), were hybridized in various pairs. Examination of autoradiographs of the hybridization products (Figure 4, A and B) and of a plot (Figure 4C) of relative migration distances of the various bands observed in Figure 4A confirmed that for each mt-tRNA gene tested there occurs in *M. californianus* mitochondria a tRNA of the size predicted from the respective gene, but to which three nucleotides, presumably CCA, have been added. In the lanes probed for tRNA^{Met}(AUA) and for tRNA^{Ser}(UCN) there is also visible a second, lighter band indicating the presence of an RNA either 2 or 3 nt smaller than the main band, a size expected for tRNAs lacking either CA or CCA at their 3′ ends.

ψtRNA^{Ser}(UCN)-containing transcripts: The observation that an oligonucleotide complementary to the ψtRNA^{Ser}(UCN) gene sequence hybridized only to RNAs

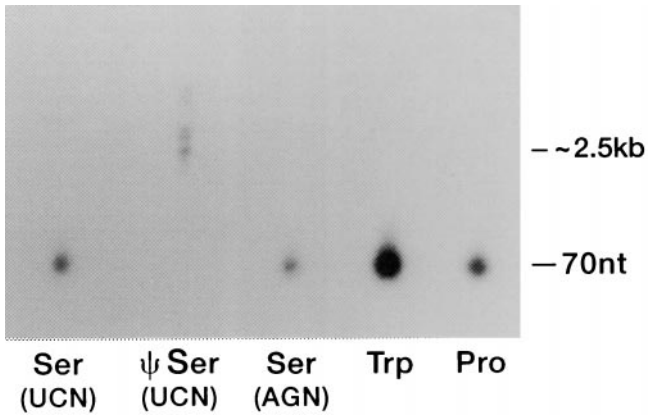

Figure 3.—Northern blot hybridization experiment to detect and determine the relative sizes of RNAs containing transcripts of genes for the DHU arm-replacement loop-containing tRNA^{Ser}(UCN), the DHU arm-replacement loop-containing tRNA^{Ser}(AGN), the four-armed tRNA^{Ser}(UCN), tRNA^{Trp}, and tRNA^{Pro} predicted from gene sequences of *M. californianus* mtDNA. In the autoradiograph shown, each lane contains 3 μg of *M. californianus* mtRNA separated by electrophoresis through a 3% denaturing (2.2 m formaldehyde) agarose gel. The locations of the tRNA^{Ser}(UCN), tRNA^{Ser}(AGN), tRNA^{Trp}, tRNA^{Pro}, and ψtRNA^{Ser}(UCN) gene-specific probes used are shown in Figure 2. Approximate band sizes (in kilobases to the right) were determined by comparison to a methylene blue-stained 0.24- to 7.46-kb ladder (Gibco-BRL; 5 μg/lane) produced by coelectrophoresis.

of ~2500 nt indicates that the ψtRNA^{Ser}(UCN) transcript remains linked to a transcript that includes the downstream COI gene and/or the upstream ND3 gene. To resolve the relationship of the ψtRNA^{Ser}(UCN) transcript and adjacent gene transcripts, Northern blot hybridizations were carried out using *M. californianus* whole mtRNA and probes consisting of an oligonucleotide complementary to the 3′ end of the ND3 gene (F3, Figures 2 and 5A), an oligonucleotide complementary to the ψtRNA^{Ser}(UCN) gene (F5, Figures 2 and 5A), and an oligonucleotide complementary to the 5′ end region of the COI gene (F4, Figures 2 and 5A). The results are shown in Figure 5B. The ND3 probe hybridized to a single band of RNA of ~350 nt, the size (351 nt) expected for an RNA containing only a transcript of this gene. However, the ψtRNA^{Ser}(UCN) probe and the COI probe both hybridized to RNA of ~2400 nt. Therefore, these data indicate that transcripts of the ψtRNA^{Ser}(UCN) sequence and the COI gene, and probably the downstream ATPase6 gene (assuming identical gene order in this region of *M. californianus* and *M. edulis* mtDNAs), are contained in the same RNA molecule.

5′ RACE analysis was used to locate the 5′ terminus of the *M. californianus* COI gene-containing transcript. cDNAs were generated from whole mtRNA using random hexamers and reverse transcriptase. A poly(A) sequence was added to the 3′ end of the cDNAs using terminal deoxynucleotidyl transferase, and cDNA complementary strands were synthesized from a 35-nt primer (TB 17-1) containing a 19-nt sequence that includes *Bgl*II, *Bam*HI, and *Pst*I sites followed by 16 T's (Pont-Kingdon *et al.* 1994). Double-stranded cDNAs of the 5′ end region of the COI-gene-containing transcript were selectively amplified in a polymerase chain reaction (PCR) using as primer oligonucleotide F4 (Figures 2 and 5B) that is complementary to a sequence within the 5′ end region of the COI transcript and a 19-nt sequence (TB 17-2) comprising the *Bam*HI-*Bgl*II-*Pst*I sequence of primer TB 17-1. The PCR product was inserted into M13mp19 DNA, and five clones of the COI transcript 5′ end region were sequenced. All five sequences obtained were identical and comparison of this sequence with the mtDNA sequence indicated that the 5′ end nucleotide of the COI gene-containing transcript is seven nucleotides downstream from the TAA termination codon of the ND3 gene (Figure 6).

These data indicate that the entire ψtRNA^{Ser}(UCN) transcript [which is separated from the 5′ terminus of the COI gene by 126 nt (Figures 1 and 5A)] remains

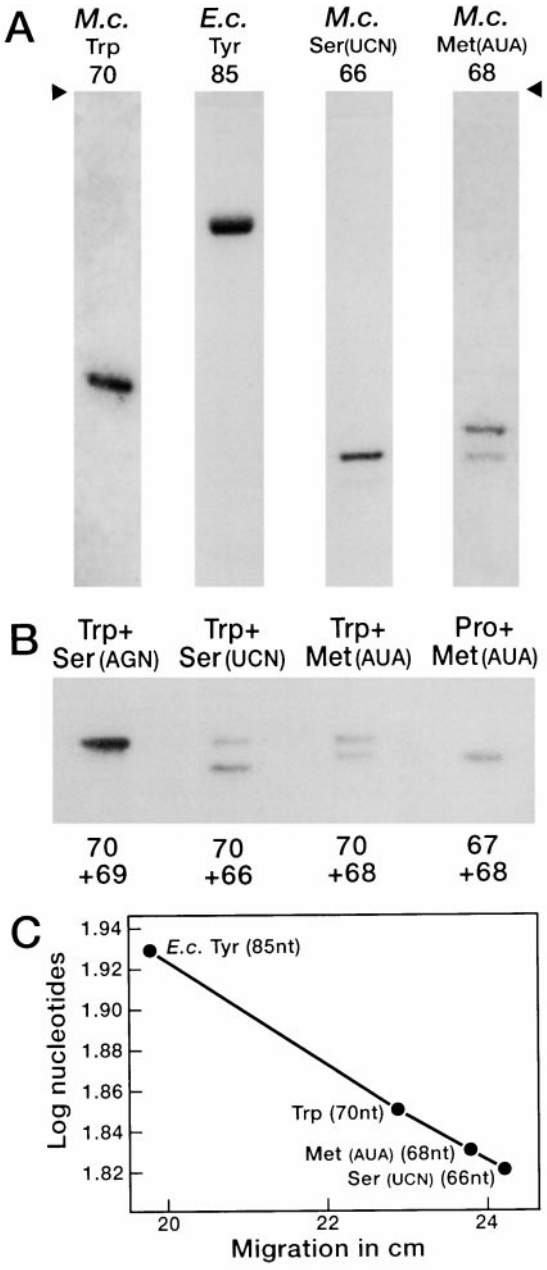

Figure 4.—(A and B) Northern blot hybridization experiments to precisely determine the sizes of tRNAs predicted from tRNA^{Trp}, tRNA^{Ser}(UCN), tRNA^{Ser}(AGN), tRNA^{Met}(AUA), and tRNA^{Pro} gene sequences of *M. californianus* mtDNA. In the autoradiographs shown, each lane contains 10 μg of *M. californianus* mtRNA (*M.c*) or (A only), as a standard, 200 ng of *E. coli* (*E.c*) tRNA^{Tyr} (85 nt including the 3′ CCA) that have been electrophoresed through a 15% polyacrylamide gel containing 8 m urea. In A, the arrowheads that bracket the top of the gels identify the common 17-cm migration position. At the top of each lane containing *M. californianus* (*M.c*) mtRNA is shown the ^{32}P-labeled tRNA probe or mixture of probes used (Figure 2). The expected number of nucleotides in the tRNA (or tRNAs) predicted from each gene sequence, plus CCA, are shown above each lane in A, and below each lane in B. The location of *E. coli* tRNA^{Tyr} was also determined by hybridization of a ^{32}P-labeled oligonucleotide complementary to that tRNA. (C) A plot of the expected size (log nucleotides) of each of the three mt-tRNAs (based on the size of the respective gene and the assumption that CCA is added post-transcriptionally at the 3′ end of each tRNA) and *E. coli* tRNA^{Tyr} (including the 3′ terminal CCA) *vs.* the measured migration distance of the tRNAs in A.

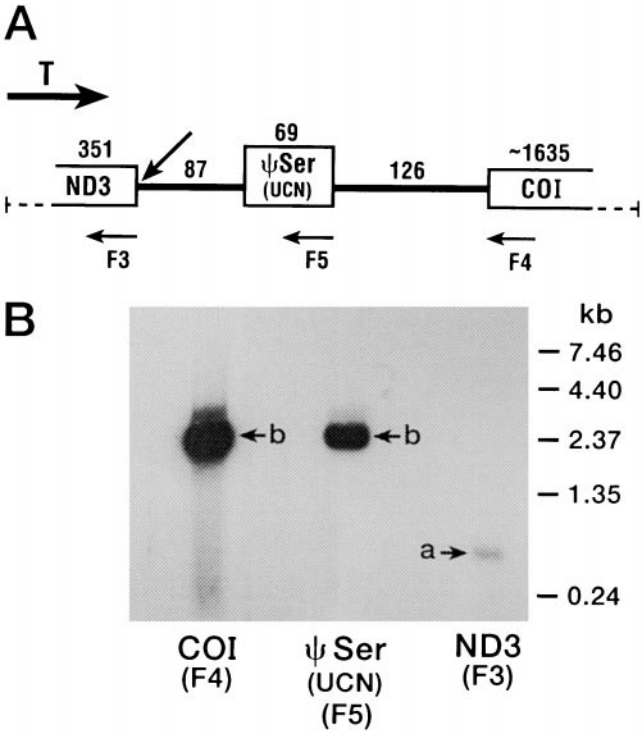

Figure 5.—Northern blot hybridization experiment to detect and determine the relative sizes of the transcripts containing the COI gene, the ψtRNA^{Ser}(UCN) gene, and the ND3 gene of *M. californianus* mtDNA. (A) Gene map to show the locations and directions of oligonucleotide probes used (arrows labeled F3 and F4, Figure 1B; and F5, Figure 2). The number of nucleotide pairs in the ND3 and ψtRNA^{Ser}(UCN) genes, and in the intergenic spaces, and the estimated number of nucleotide pairs (1635) in the COI gene are shown. (B) Autoradiographs of Northern blots. Each lane contains 3 μg *M. californianus* mtRNA separated by electrophoresis through a 1% denaturing (2.2 m formaldehyde) agarose gel. The COI, ND3, and ψtRNA^{Ser}(UCN) gene-specific oligonucleotides used to probe each lane are shown. Sizes of bands (arrow a, 0.35 kb; arrows b, 2.5 kb) were determined by comparisons to a methylene blue-stained, 0.24- to 7.46-kb ladder (5 μg/lane; Gibco-BRL; represented in kilobases to the right).

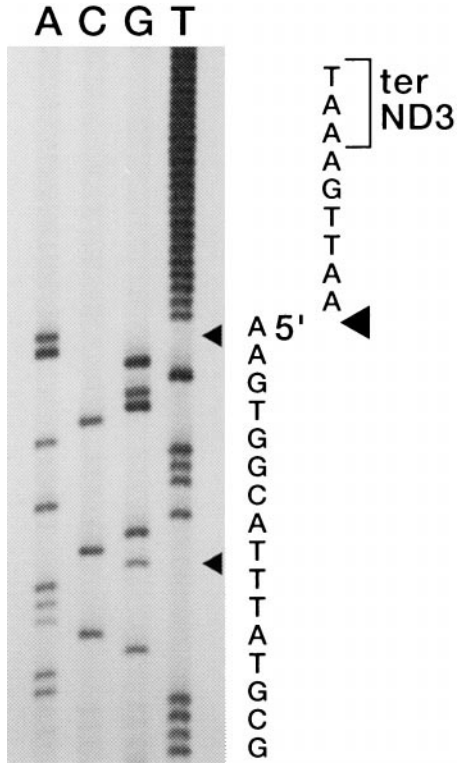

Figure 6.—Autoradiograph showing the nucleotide sequence of a clone of the amplification product of a 3′ polyadenylated (using terminal deoxynucleotidyl transferase) cDNA of the 5′ end-proximal region of the *M. californianus* mitochondrial transcript that contains the ψtRNA^{Ser}(UCN) and COI genes. As the sequence shown is the transcript equivalent, the terminal reiterated nucleotide is T rather than A. The upper small arrowhead indicates the 5′ terminal nucleotide of the transcript. To the right is shown the sequence downstream from the poly-T sequence (equivalent to the sequence between the two small arrowheads). The written sequence above the large arrowhead is that between the termination codon of the ND3 gene (ter ND3) and the 5′ end of the ψtRNA^{Ser}(UCN)-COI gene transcript.

linked to the COI transcript. The finding that the 5′ end of the COI-gene-containing transcript is 81 nt upstream from the 5′ end of the ψtRNA^{Ser}(UCN) sequence makes it unlikely that there has been selection for retention of the tRNA^{Ser}(UCN) sequence so that its secondary structure can act as a transcript processing site, a role suggested for interprotein gene tRNA sequences in mammalian multicistronic mtDNA transcripts (Ojala *et al.* 1981). Interestingly, however, in this regard the 5′ end region of the *M. californianus* COI gene transcript can be folded into a stable stem and loop structure that includes the transcript cleavage site. This stable secondary structure potential is conserved in the corresponding sequence of *M. edulis* (F-type) mtDNA (Figure 7). Similar (but smaller) potential single stem and loop structures have been identified at the 3′ ends of protein genes that are immediately followed by another protein gene in mtDNAs of a number of metazoan species including mouse, *D. yakuba*, *C. elegans*, and *A. suum*, and it has been suggested that these sequences may be important for precise transcript cleavage (Bibb *et al.* 1981; Clary and Wolstenholme 1985; Okimoto *et al.* 1992). Hoffmann *et al.* (1992) noted secondary structure potential of both the sequence between the ND3 and ψtRNA^{Ser}(UCN) genes and the sequence between the ψtRNA^{Ser}(UCN) and COI genes in *M. edulis* mtDNA, but they concluded that the secondary structure of the first of these sequences was nonsignificant.

CONCLUDING REMARKS

In metazoan mtDNAs, although most adjacent genes are separated by few or no nucleotides, there is a single region of apparently noncoding nucleotides that varies in size between 125 ntp and ~8000 ntp. As this region in both mammalian and Drosophila mtDNAs has been

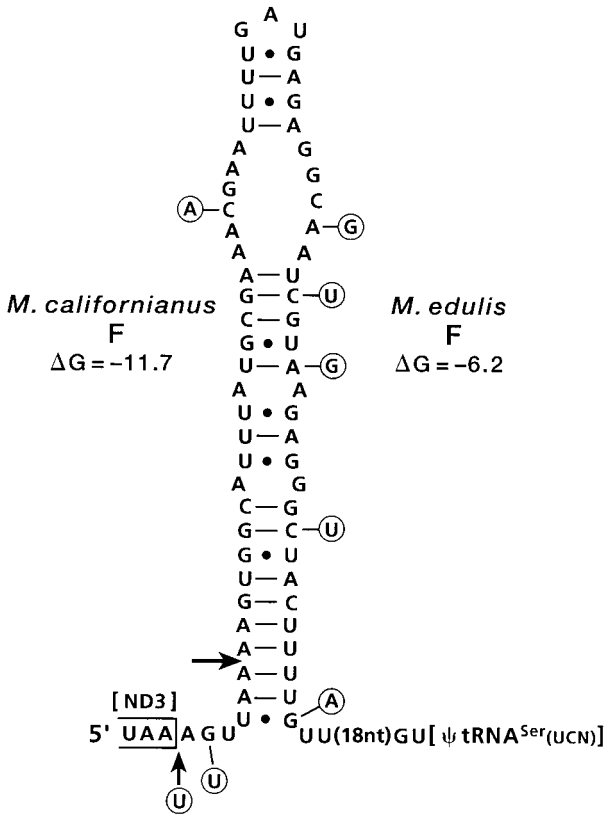

Figure 7.—Potential stem and loop configuration of the nucleotide sequence of the 5′ end region of the COI transcript and presumed 3′ end region of the ND3 transcript of F-type *M. californianus* mtDNA. The 5′ end of the COI transcript determined by 5′ RACE analysis (Figure 6) is indicated by an arrow. Transcription is from left to right. The ND3 translation termination codon is boxed. The location of the ψtRNA^{Ser} (UCN) sequence within the COI transcript is shown. Circled nucleotides are substitutions in the corresponding transcripts of F-type *M. edulis*, predicted from the mtDNA. A nucleotide present in the *M. edulis* sequence but not in the *M. californianus* sequence is indicated by an arrow attached to a circled U. The predicted free energy changes shown were calculated using the free energy increments given by Freier *et al.* (1986).

shown to contain the molecule's replication origin, and also in mammalian mtDNAs to contain promoters for transcription of multicistronic RNAs, it has been designated the control region (Montoya *et al.* 1981; Clayton 1982, 1992; Shadel and Clayton 1997). The larger size of some control regions of a variety of Metazoa result from the presence of repeated sequences of different sizes and numbers (for references see Wolstenholme 1992a). Cases have been reported of a repeated segment of mtDNA that contains whole or partial tRNA, rRNA, and protein genes. These include mtDNAs of different Cnemidophorus lizard species, the gecko *Heteronotia binoei* (Moritz and Brown 1986, 1987; Moritz 1991), the newt *Triturus cristatus* (Wallis 1987), and the nematode *Romanonermis culicivrax* (Hyman *et al.* 1988). However, whether or not any of these repeated genes might have a function has not been examined. The presently provided evidence that a sequence that appears to have acted as a tRNA^{Ser}(UCN) gene at some time in the past is now nonfunctional (at least in its original capacity) is the first direct demonstration of a pseudogene in metazoan mtDNA.

The single functional tRNA^{Ser}(UCN) gene of the two Mytilus species appears to be a 63-nt sequence located between the COIII and tRNA^{Met}(AUA) genes, which encodes a highly conserved DHU-replacement loop-containing tRNA. The interrelationship of the functional tRNA^{Ser}(UCN) gene and the ψtRNA^{Ser}(UCN) gene remains unclear. Sequence comparisons do not suggest that one is a duplicate of the other. As there is considerable divergence between the *M. californianus* and *M. edulis* mt-ψtRNA^{Ser}(UCN) gene sequences it remains possible that these sequences are the remnants of a gene for a tRNA other than one that recognizes UCN codons. However, this is not supported by sequence comparisons to other *M. edulis* mt-tRNA genes. It has been suggested that the tRNA^{Ser}(UCN) genes of the gastropods *Euhadra herklotsi* and *Cepaea nemoralis* (but not *Albineria coerulea*), the polyplacophoran mollusk *Katherina tunicata*, and the annelid *Lumbricus terrestris* encode DHU arm-replacement loop-containing structures (Boore and Brown 1994, 1995; Hatzoglou *et al.* 1995; Terrett *et al.* 1996; Yamazaki *et al.* 1997). However, the *K. tunicata* and *L. terrestris* predicted tRNAs can be folded to give the usual U_8, A_9, or G_9 between the aminoacyl and DHU arms, and a DHU arm with a stem of 3 ntp and a loop of 3 nt, but with three (rather than the usual one) nucleotides between the DHU and anticodon arms. Comparisons of the DHU arm-replacement loop-containing tRNA^{Ser} (UCN) genes of the two Mytilus species and the tRNA^{Ser} (UCN) gene of *K. tunicata* reveal conservation of 4/5 ntp in the TψC stems, 3/5 ntp in the anticodon stems, and 7/7 nt in the anticodon loops.

The mt-tRNA^{Ser}(UCN) genes of arthropods, echinoderms, and chordates so far examined encode a four-armed tRNA and the primary sequences of these tRNAs of the latter two phyla are close to those of standard tRNAs. However, as in mtDNAs of mollusks and an annelid, genes for tRNAs expected to recognize UCN codons that have a DHU arm-replacement loop have also been found in nematode mtDNAs (Okimoto and Wolstenholme 1990; Okimoto *et al.* 1992). Considerations of the various proposed relationships of invertebrate phyla (see, for example, Wilson *et al.* 1978; Nielsen 1995) do not lend support to the view that the observed distribution of the genes for the two structural kinds of mt-tRNA^{Ser}(UCN) could have resulted from a single loss of a DHU arm-forming sequence from a mt-tRNA^{Ser}(UCN) gene during invertebrate evolution. Because it seems improbable that, once lost, a DHU arm-forming sequence of standard tRNAs would be regenerated (discussed in Wolstenholme *et al.* 1987), then the most likely explanation for the presence of a DHU arm-replacement loop-containing mt-tRNA^{Ser}(UCN) in nem-

atodes, some (but not all) mollusks, and an annelid, is that the gene for this kind of tRNA has been generated by multiple independent sequence losses. This would represent an unusual case of convergent structural evolution in metazoan mt-genomes.

The mtDNA sequence interpreted by Hoffmann *et al.* (1992) as an *M. edulis* $tRNA^{Met}$(AUA) gene is highly conserved in *M. californianus* mtDNA. Our finding that an RNA molecule of the sequence and size expected for a mature tRNA encoded by the *M. californianus* $tRNA^{Met}$(AUA) gene occurs in *M. californianus* mitochondria provides strong evidence that this gene is functional.

Hoffmann *et al.* (1992) stated that AUA specifies methionine in the *M. edulis* mt-genetic code, but they did not explain how they reached this conclusion. In fact, comparisons of ATA codons in the partial sequences of *M. edulis* Cyt b, COI, and COII genes (more highly conserved genes in metazoan mtDNAs), with similarly located codons in *C. elegans*, *D. yakuba*, and mouse mtDNAs, do not provide compelling evidence that AUA is more likely to encode methionine than isoleucine or leucine. However, interchange of ATA and ATG codons in the partial sequences of *M. californianus* F-type and M-type COI and ND5 genes (Beagley *et al.* 1997) does strongly support the coding specificity of AUA as methionine. Of 15 ATA codons that occur in these M-type sequences, 6 have ATA and 7 have ATG in the corresponding locations of the F-type sequences; of 8 ATG codons that occur in the M-type sequences, 3 have ATG and 4 have ATA in the corresponding locations of the F-type sequences. Therefore, assuming that the $tRNA^{Met}$(AUG) gene is functional, which seems likely, as suggested by Hoffmann *et al.* (1992), Mytilus mtDNAs are unique among known metazoan mtDNA sequences in that they contain two methionine-specifying tRNAs. Based on sequence similarities in the aminoacyl and DHU arms, Hoffmann *et al.* (1992) suggested that the $tRNA^{Met}$(AUA) gene may have arisen as a duplication of the $tRNA^{Met}$(AUG) gene.

It is uncertain why Mytilus alone among Metzoa examined to date should utilize two tRNAs to recognize methionine codons. In *M. edulis*, the $tRNA^{Met}$(AUG) would be expected to recognize the AUG initiation codons of 10 of the 12 mt-protein genes and internal AUG codons. In regard to its initiation function it is noteworthy that the predicted tRNA would have three consecutive G-C base pairs at the base of the anticodon stem. This is a characteristic feature of the *E. coli* initiator $tRNA^{Met}$ and it has been shown that these three G-C base pairs are essential to initiation function (Rajbhandary and Chow 1995). Also, three, two, or one of these G-C nucleotide pairs occur in the single mt-$tRNA^{Met}$ of various other Metazoa (Anderson *et al.* 1982; Clary *et al.* 1982; Wolstenholme *et al.* 1994; Beagley *et al.* 1995). All but one of the ATA codons in the *M. edulis* mtDNA molecule are internal. Only in the ND5 gene does it appear likely that the initiation codon is ATA. We have been unable to detect any convincing similarity between the Mytilus $tRNA^{Met}$(AUA) gene and the *E. coli* elongator $tRNA^{Met}$ that recognizes only internal AUG codons.

We thank Jane L. Macfarlane for excellent technical assistance and for discussions, and Robert Schackmann for oligonucleotides [partially subsidized by National Institutes of Health (NIH) grant CA-42014]. This work was supported by NIH grant GM-18375.

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Communicating editor: G. B. Golding