Identification of the 3'-Ends of the Two Mouse Mitochondrial Ribosomal RNAs

The 3'-termini of the mitochondrial 12 S and 16 S ribosomal RNAs from mouse L cells have been definitively characterized by mobility-shift RNA sequencing, RNase digestion followed by fingerprinting using two-dimensional homochromatography, and precise mapping of RNA-DNA duplexes using nuclease S1. The results have been correlated with the known DNA sequence of the rRNA region. The vast majority of the 12 S rRNA consists of a family of transcripts whose last template-encoded nucleotide corresponds to a position immediately adjacent to the 5'-end of the tRNAal gene in the DNA sequence. These transcripts are oligoadenylated at their 3'-ends with from 1 to about 5 adenylate residues that are not encoded in the DNA sequence. A minor proportion of the 12 S rRNA ends one nucleotide before the 12 S/rRNA™ gene boundary and is also oligoadenylated. In contrast, the 3'-termini of 16 S rRNA have considerably greater heterogeneity, with the genomic location of the last template-encoded nucleotide varying from the nucleotide immediately adjacent to the 5'-end of the tRNAal gene in any direction up to 7 nucleotides downstream within the tRNAal™ gene sequence. These various 16 S rRNA transcripts are oligoadenylated to a somewhat greater degree than the 12 S rRNA. The extent of the 16 S rRNA 3'-heterogeneity, as compared to the 12 S rRNA, suggests that the 16 S rRNA 3'-termini may be generated by a mechanism involving termination of transcription rather than by processing of a primary transcript. The data are similar to those reported for human mitochondrial rRNA 3'-termini, and support a general role for adenylation of 3'-termini in the termination or processing of mammalian mitochondrial RNAs.

The mammalian mitochondrial genome displays exceptional economy of organization with tRNA genes interspersed between rRNA and protein-coding genes with zero or few noncoding nucleotides between coding sequences (1). The lack of noncoding sequence between the various genes suggests that the entire genome may be transcribed as a polycistronic precursor RNA from which individual tRNAs, rRNAs, and mRNAs are generated by single endonucleolytic cleavages at the 5' and 3' borders of RNA sequences. The mitochondrial mRNAs are subsequently polyadenylated with from 50 to 60 A residues (2). Great accuracy is required in cleavage of such a precursor transcript to ensure that the mature RNAs will be biologically functional. For example, 6 of the 13 protein-coding genes in mouse mitochondria do not have translational termination codons encoded in the DNA (1). Transcripts from these genes require precise cleavage at the gene boundary after U or UA, followed by polyadenylation, in order to form a functional UAA termination codon (1).

The assignment of gene boundaries for the small and large mouse mitochondrial rRNAs has been made using direct sequencing of the 5'- and 3'-ends of the rRNAs, together with location of tRNA genes by secondary structures predicted from the DNA sequence (3) (Fig. 1). Analysis of the 5'-ends of the 12 S and 16 S rRNAs demonstrated unique 5' termini which map adjacent to the tRNA™ and tRNA™ genes, respectively, placing one or no noncoding nucleotides between the tRNA and rRNA genes at these boundaries. While a sequence obtained from the 3'-terminus of the 12 S rRNA was interpreted to indicate termination of the rRNA at the predicted gene boundary immediately adjacent to the 5'-end of the tRNA™ gene, a unique sequence of the 3'-end of the 16 S rRNA could not be obtained, suggesting considerable 3'-terminal heterogeneity. Recently, the 16 S rRNA species from hamster (4) and human (5) mitochondria have been shown to have 3'-terminal heterogeneity, with variable transcription termination or processing, as well as post-transcriptional oligoadenylation. We have used a variety of RNA sequence analyses and RNA-DNA mapping experiments to precisely characterize the unusual nature of the 3'-termini of the mouse mitochondrial rRNAs and to correlate the results with the corresponding gene sequences obtained from the complete DNA sequence of the mouse mitochondrial genome (1).

**ExPERIMENTAL PROCEDURES**

Preparation, labeling, and characterization of mitochondrial RNA — isolation of rRNA from L cell and mouse liver mitochondria (16). The mitochondrial RNA from L cell and mouse liver mitochondria was isolated by hot phenol extraction of a protease-digested pellet as described by Van Etten et al. (16). The RNA was labeled at 3'-ends with [32P]PNAS using RNA ligase (16). The original mitochondrial RNA was also labeled at the 5'-ends with [32P]PNAS using RNA ligase (16). The RNA ligase reaction was performed in the presence of RNA and RNA ligase (16). The RNA ligase reaction was performed in the presence of RNA and RNA ligase (16). The RNA ligase reaction was performed in the presence of RNA and RNA ligase (16). The RNA ligase reaction was performed in the presence of RNA and RNA ligase (16). The RNA ligase reaction was performed in the presence of RNA and RNA ligase (16).

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**Structure of the rRNA region of mouse mtDNA.** Location of 12 S and 16 S rRNA genes and tRNA genes are as discussed in the text and Ref. 3. URFl is a gene for an unidentified protein. The D-loop region and location of restriction sites utilized in S1 protection experiments are indicated. Scale denotes distance in kilobase pairs; numbers in parentheses indicate the number of intervening nucleotides between 3' ends of tRNA genes and 5' ends of rRNA genes or the initiation codon of URFl, respectively.

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**Results**

12 S rRNA 3′-Termini—3′-End labeled 12 S rRNA was highly purified by hybrid selection (8) as described under "Experimental Procedures," and the nature of the 3′-terminal nucleotide prior to end labeling was determined by digestion of a sample to completion with RNase T2 followed by thin layer chromatography. This analysis yielded >95% Ap (data not shown) in agreement with previous work (3). Mobility-shift sequence analysis of partially hydrolyzed 12 S rRNA is shown in Fig. 2 and yields a major component with sequence 5′-UUGGAAUAAUAC-3′ where the 3′-terminal C is added during the labeling reaction. The spot at the lower right is the dinucleotide ApCp, a consequence of the fact that it is the penultimate 3′-phosphate and not the terminal 3′-phosphate which is radiolabeled. This sequence corresponds precisely to the sequence at the predicted gene boundary between the 12 S rRNA and tRNAVal (3) except that a single non-template-encoded A residue is present at the 3′-end. It is now clear that the previous 12 S rRNA 3′-terminal sequencing gel autoradiogram (3) was misinterpreted, and that both experiments indicate that the majority of 12 S rRNA terminates with an A residue which is presumably added post-transcriptionally to the U residue encoded at the 3′-end of the gene. In addition, the mobility shift analysis revealed several other series of spots of lower abundance, indicating the presence of minor heterogeneity of the 12 S rRNA 3′ terminus.

Samples of 3′-end labeled 12 S rRNA were then subjected to complete digestion with RNase T1 and the 3′ terminal T1 and T2 enzymes.
oligonucleotides were “fingerprinted” using two-dimensional homochromatography as described under “Experimental Procedures” (Fig. 3). Two tracts of spots are visible, with the majority of the radioactivity present in spot 4. Secondary analysis of this spot showed that its 3’-terminal sequence was YAC. The other minor spots immediately above spot 4 in Fig. 3 were shown to differ from spot 4 by successive addition of single A residues at the 3’-end. The other tract of spots in Fig. 3 is displaced to the right, in a position consistent with these spots differing from spots 4 through 7 by the loss of a U residue. Secondary analysis of spot 1 revealed that its 3’-terminal sequence was YAAAC. The minor spots above spot 1 in Fig. 3 again differ in sequence by single A residues at the 3’-end.

When these results are compared with the DNA sequence of the 12 S rRNA/tRNA\textsuperscript{val} gene boundary, it is seen that the 12 S rRNA species that generated spot 4 is the same one responsible for the major series of spots seen on the mobility-shift analysis, and corresponds to a 12 S RNA which is monoadenylated after the 3’-terminal U. Spots 5 through 7 represent similar termini with higher degrees of oligoadenylation. The most probable origin of the minor tract in Fig. 3 is from termini of the form ...GAAUAAA, such that spot 1 corresponds to a 12 S species which terminates one nucleotide prior to the U at the 12 S rRNA/tRNA\textsuperscript{val} gene boundary and also has a single A residue added post-transcriptionally. These data are summarized in Fig. 4 and Table I. The major 12 S rRNA species, comprising about 66% of the total, represents monoadenylation of a transcript ending at the predicted gene boundary, with minor amounts of higher order oligoadenylation. A second minor family consists of transcripts ending one nucleotide before the gene boundary, also with oligoadenylation.

To confirm the placement of the 12 S rRNA 3’-termini at the predicted gene boundary, an S1 nuclease protection experiment was performed. A cloned mtDNA restriction fragment was 3’-end labeled at an XbaI site mapping approximately 60 nucleotides upstream of the 12 S rRNA/tRNA\textsuperscript{val} gene boundary as described under “Experimental Procedures.” This restriction fragment was then annealed to 12 S rRNA and treated with the single strand-specific nuclease S1. RNA-protected DNA strands were precisely sized by fractionation on an 8% polyacrylamide-urea sequencing gel, adjacent to DNA sequencing lanes generated by the Maxam-Gilbert chemical procedure (15) applied to the same 3’-end labeled DNA fragment used for S1 protection (Fig. 5). Because the chemical cleavage reactions destroy the nucleotide at which base-specific strand scission occurs, the actual sequence of a polynucleotide corresponding to a band in a DNA sequence ladder is one shorter than the sequence which is read from that position in the ladder. With this correction, it was found that under the hybridization conditions used, an S1 concentration of between 10,000 and 20,000 units/ml yielded the major S1-protected species at the expected position at the gene boundary between the 12 S rRNA and tRNA\textsuperscript{val} genes. At this concentration of S1, there is not a single S1-protected species nor a pair of species corresponding to the two families of transcripts, but rather a series of 6 to 7 bands extending several nucleotides downstream, which may reflect steric hindrance of the enzyme by non-basepaired adenylate residues present at the 3’-end of the RNA/DNA hybrid. Higher concentrations of S1 do not reduce this heterogeneity but result in reduction in size of the major protected species, indicating “nibbling” of S1 past the end of the RNA/DNA duplex. Therefore, nuclease protection experiments of this kind cannot be used to make precise assignment of 3’-termini at the nucleotide level, but the results confirm the placement of the 12 S rRNA 3’-termini to the predicted gene boundary.

16 S rRNA 3’-Termini—Highly purified 3’-end labeled 16 S rRNA was isolated as described for 12 S rRNA. 3’-End analysis with RNase T\textsubscript{2} revealed >90% Ap with minor amounts of Up (data not shown). Mobility-shift analysis of the 16 S rRNA, however, gave too many spots to yield any interpretable sequences (data not shown). Samples of 3’-end labeled 16 S rRNA were digested to completion with RNase A or RNase T\textsubscript{1} and fingerprinted using two-dimensional homochromatography. The fingerprint of the 3’-terminal RNase A oligonucleotides is shown in Fig. 6, and shows a predominant tract of spots on the right side. These spots, eluted and analyzed by paper electrophoresis, were shown to be (from

![Fig. 3](https://image-url.com/fig3.png)

**Fig. 3.** Two-dimensional homochromatography of RNase T\textsubscript{1} digest of 12 S rRNA. Orientation and dye marker are as described for Fig. 2. Secondary analyses of spots were as described under “Experimental Procedures.” Spot 1, AAUAACP; spot 2, AUAACP; spot 3, AAUAACP; spot 4, AAUAUAACP; spot 5, AAUAUAACp; spots 6 and 7, AAUAUUACp. On secondary analyses spot 4 yielded ACP after RNase A digestion and pH 3.5 electrophoresis and spot 1 yielded ACP after RNase A digestion and pH 3.5 electrophoresis (data not shown).

![Fig. 4](https://image-url.com/fig4.png)

**Fig. 4.** Schematic representation of 3’-terminal template-encoded nucleotides of the 12 S and 16 S rRNAs. 5’-Ends of tRNA\textsuperscript{val} and tRNA\textsuperscript{val} genes are indicated by the boxes. Arrows specify the 3’-terminal template-encoded nucleotides of the rRNAs; the thickness of the arrows qualitatively represents the relative abundances of the various termini. Dots below A residues indicate that termination at these positions cannot be distinguished from termination one nucleotide earlier followed by oligoadenylation (see text).
Radioactivity from individual spots on fingerprints was determined by cutting the thin layer into sections and assaying each in a scintillation counter. A schematic representation of the template-encoded portion of the 3' termini of 12S and 16S rRNA is shown, along with the relative abundance of each terminus and the extent of oligoadenylation at each position.

| Template-encoded portion | Total | 0 | 1 | 2 | 3 | >3 | 4 | 5 | 6 | 7 | 8 | >8 |
|--------------------------|-------|---|---|---|---|----|---|---|---|---|---|----|
| 12S rRNA                 |       |   |   |   |   |    |   |   |   |   |   |    |
| ...UUGGAAUAU             | 92.5  | <0.1| 65.8| 11.2| 10.2| 5.3 |   |   |   |   |   |    |
| ...UUGGAAUAA             | 7.5   | <0.1| 4.3 | 2.4 | 0.7 | 0.1 |   |   |   |   |   |    |
| 16S rRNA                 |       |   |   |   |   |    |   |   |   |   |   |    |
| ...GGGUU                 | 68.3  | 1.7 | 7.9 | 6.8 | 6.6 | 6.1 | 8.1 | 6.8 | 5.0 | 12.2 |    |
| ...GGGUUAU               | 8.9   | <0.1| 0.2 | 0.2 | 1.5 | 0.8 | 2.3 | 1.5 | 0.9 | 1.5 |    |
| ...GGGUUAUU              | 1.3   | <0.1| 0.2 | 0.7 | 0.2 | 0.2 |    |    |    |    |    |
| ...GGGUUAUAG             | 8.1   | <0.1| 2.3 | 2.3 | 2.0 | 0.7 | 0.8 |    |    |    |    |
| ...GGGUUAUAGG            | 12.3  | <0.1| 5.0 | 2.2 | 1.2 | 0.9 | 1.0 | 2.0 |    |    |    |
| ...GGGUUAUAGGG           | 0.7   | <0.1| 0.5 | 0.2 |    |    |    |    |    |    |    |

the bottom up) AC, AAC, AAAC, etc., indicating that the majority of 16 S rRNA species are oligoadenylated after a pyrimidine. Three other minor tracts of spots are also present to the left of this main tract. End analysis (using RNase T2) showed the penultimate nucleotide of each of these spots to be A. Spots 9 and 14 were analyzed by RNase T1 digestion and paper electrophoresis yielding AC for the 3'-terminal sequence of both spots. The sequence of spot 9, which is a tetranucleotide, is therefore of the form RGAC, and by its position on the fingerprint must be AGAC. The positions of spots 14 and 20 are consistent with these oligonucleotides differing from spot 9 by the addition of 1 and 2 G residues, respectively. Thus the sequences of spots 14 and 20 are (AG)GAC and (AGG)GAC, respectively. The upper series of spots in each family once again represents oligonucleotides which differ from the next lower spot by addition of single A residues at the 3'-end.

There are four visible tracts of spots on the fingerprint of an RNase T1 digest of the 3'-end labeled 16 S rRNA (Fig. 6B). The rightward tract is an A,C (n = 1, 2, 3,...) series similar to that seen in the RNase A fingerprint, and represents oligoadenylation of 16 S rRNA after a G. The three other tracts are shifted well over to the left, indicating the presence of one or more U residues in these oligonucleotides. Spots 9, 10, 11, and 12 from the major tract were analyzed by complete digestion with either RNase T2 or nuclease P1, which indicated that all spots had C as the 3'-terminal nucleotide and A as the penultimate nucleotide, except for spot 9, which had U as the penultimate nucleotide. RNase A digestion of spot 10 yielded radiolabeled AC. Spot 10 was analyzed on paper electrophoresis without further digestion and found to have a mobility consistent with the sequence UAUC, rather than UAC or UUUAC. Therefore this tract of spots represents a family of 16 S rRNA transcripts with 3'-terminal sequences, after labeling, of the form ...GUUA,U,C (where n = 0, 1, 2, 3,...). The other two tracts had too little radioactivity for direct secondary analysis, but their position on the fingerprint is consistent with them differing from the main tract by 1 and 2 additional U residues.

Examination of the mouse mtDNA sequence in the vicinity of the 5'-end of the tRNAVal gene (Fig. 4B) shows that the boundary is indicated. Reactions shown utilized crude mtRNA as described under "Experimental Procedures"; reactions with electrophoretically purified 12S rRNA gave identical results; control reactions without RNA gave no bands (data not shown).
major family of 16 S transcripts terminates at the nucleotide prior to the 5'-end of the tRNA^{16S} gene, with considerable oligoadenylation generating the longer members of the family. By correlation with the DNA sequence, the minor tracts of spots on the 16 S RNA A and T1 fingerprints can be unambiguously assigned to 16 S transcripts with oligoadenylation terminating one nucleotide before the 16 S rRNA gene. Two other sets of bands are also present, mapping 15 to 20 nucleotides within the tRNA gene. These species are thought to be artifacts, based on the fact that they disappear with increasing S1 concentration, they appear in reduced relative intensity when the RNA:DNA ratio in the hybridization reaction is increased, and 16 S RNA with these termini would generate distinctive spots on the RNase T1 fingerprint which are not seen.

The data from the fingerprints and the S1 protection experiment are summarized in Fig. 4B and Table I. The majority (>65%) of the 16 S RNA terminates at the gene boundary, and is extensively oligoadenylated, yielding RNAs with a 3'-terminal sequence after labeling of ...GGUUA,A,C (n = 0, 1, 2, 3,...). The remainder of the termini represent minor termination events from 1 to 7 nucleotides into the downstream tRNA^{16S} gene, which are also oligoadenylated. The leftmost two tracts of the 16 S rRNA RNase T1 fingerprint are now identified as originating from termini of the form ...GGUUA,A,C and ...GGUAAUA,C, respectively, resulting from termination and oligoadenylation just within the 5'-end of the tRNA^{16S} gene, while the tracts taken from leftward tRNA gene. We note that it is not possible to distinguish which one nucleotide before the
**DISCUSSION**

**Nature of the Ribosomal RNA 3’-Termini**—The precise characterization of the 3’-termini of the mouse mitochondrial rRNAs completes the detailed transcription map of this region of the mouse mtDNA genome and confirms the assignment of gene boundaries in this region that were previously placed by inference. Earlier work (3) had placed the 3’-end of the 16 S rRNA gene, with only one out of -50 transcriptional events proceeding beyond this point into the protein-coding gene region. In contrast, while the majority of the 16 S rRNA gene is immediately adjacent to the 5’-end of the tRNA gene partly by analogy to the 12 S/tRNA gene boundary, these rRNAs display a greater degree of 3’-end heterogeneity and post-transcriptional oligoadenylation. The phenomenon of oligoadenylation of mammalian mitochondrial rRNAs appears to be a general one, as similar patterns of 3’-termini have been observed for both hamster (4) and human (5) mitochondrial rRNAs. In those studies, the large subunit rRNA was also found to have heterogeneous template-encoded lengths and a variable degree of oligoadenylation, but exact assignment of the template distribution of the minor termini was hindered by lack of hamster mtDNA sequence data, and, in the case of the human 16 S rRNA, by ambiguities which arise in assignment of minor 3’-terminal RNase A or T1 oligonucleotides to specific DNA sequences. Due to the redundant nature of the sequence around the 3’ terminus of the human 16 S rRNA gene, it was not clear whether these minor termini were shorter than those transcripts which terminated at the gene boundary or whether they extended beyond the formal end of the gene. The fingerprint data shown above establish the major mouse mitochondrial 16 S rRNA 3’ terminus to be of the form ...GUU. A search of the mouse mitochondrial sequence for 50 nucleotides on either side of the 5’-end of the tRNA gene (Fig. 4R) demonstrated that in this system the “raggedness” of the transcript completed the detailed transcription map of this region. In those studies, the large subunit rRNA transcripts that terminate upstream of the gene boundary, demonstrating that in this system the “raggedness” of the transcribed portion of the 16 S rRNA population is due to transcripts which extend beyond the gene boundary, ranging up to 7 nucleotides into the downstream rRNA gene (Fig. 4B). It should be emphasized that these studies were performed on the total steady state population of rRNA sequences in the cell. It is not known whether a particular subclass of rRNA transcripts is specifically biologically active at the expense of others by preferential incorporation into mature mitochondrial ribosomes nor is it known what function or effect any “raggedness” or oligoadenylation of the rRNAs may have regarding interactions at the ribosomal level. Isolation of intact ribosomal subunits prior to RNA extraction and fingerprinting may help to address this question.

**Synthesis and Possible Significance of Variable 3’-Termini**—The rRNAs are the most abundant mitochondrial RNAs in the cell, and mouse mitochondria have been shown to contain approximately 50 times more rRNA than various mRNAs in the steady state (16). Available kinetic data in the human mitochondrial system suggest that this difference in abundance largely reflects differences in the rates of transcription of the rRNA gene region versus the mRNA gene regions of mtDNA (17). This suggests that there may be complete termination of transcription at the end of the 16 S rRNA gene, with a lower frequency of transcription initiation at a second promoter downstream of the 16 S rRNA gene for transcription of the remaining heavy strand-encoded RNAs. Alternatively, there may be attenuation of the majority of transcriptional events at a site near the 3’-end of the 16 S gene with only one out of ~50 transcriptional events proceeding beyond this point into the protein-coding gene region. The different degree of 3’ heterogeneity and oligoadenylation displayed by the 12 S rRNA and 16 S rRNA argues that the processes involved in their creation, although seemingly related, may be mechanistically distinct. While the 3’-terminus of mature 12 S rRNA would appear to be the product of a relatively precise cleavage of a primary transcript at the 12
S/\textit{tRNA}^{Val}\text{ border, the more extreme heterogeneity of the last template-encoded nucleotide of the 16 S rRNA seems inconsistent with these termini being generated by the same kind of precision event needed to generate the 3' terminus of a functional mitochondrial mRNA. The degree of heterogeneity of the 16 S rRNA suggests that the process which generates the 3'-end is one of termination of transcription, rather than cleavage of a primary transcript at the border with \textit{tRNA}^{16S} sequences. A mechanism involving attenuation of transcription of an imprecise or variable nature would yield the elongated 16 S rRNAs that have been described, while relief of attenuation or continuation of transcription would yield a correctly shortened 16 S rRNA and an intact \textit{tRNA}_{\text{Leu}}. Sequence determination of the mouse mitochondrial rRNA plate position 6 nucleotides downstream from the 5'-end of 16 S rRNA suggests that \textit{tRNA}^{16S} terminates at the gene boundary and is not adenylated (1.7%, Table I) closely matches the approximate proportion of 16 S termini expected to be generated by “read through” transcriptional events (∼1/50). It is possible that these nonadenylated termini represent the products of continuation of transcription past the attenuation region, while all termini which extend past the 5'-end of \textit{tRNA}^{16S} represent attenuation events. Under this model, oligoadenylation of the form ...GUAA,... would result from attenuation at the initial 5' A residue of the \textit{tRNA}^{16S} gene followed by oligoadenylation; as pointed out earlier, this cannot be distinguished on the basis of fingerprints alone from termination at the gene boundary followed by oligoadenylation.

While there is no currently available experimental system to distinguish between attenuation or complete termination in this region of the genome, the existence of a secondary promoter at or near this site seems unlikely due to the paucity of noncoding nucleotides between genes. This is in contrast to the displacement loop region, which is the only portion of the genome of significant size that contains candidate control elements (18) and which has been inferred to be the site of initiation of both heavy and light strand transcripts (19, 20). The 3'-terminal region of the 16 S rRNA gene is not particularly homologous to the sequences of other large subunit rRNA genes from various species and although several alternate secondary structures involving this region and the adjacent \textit{tRNA}^{16S} sequences can be drawn (5), there are no striking homologies with termination or attenuation sequences that have been characterized in procaryotic and eucaryotic systems. If attenuation is occurring in this region, then it is worth noting that the process of stopping transcription near the end of the 16 S gene does not involve a smooth dissociation of the polymerase from the DNA template after passing the end of the 16 S rRNA gene. Rather than exhibiting a decreasing gradient of termination frequencies as the rRNA continues further into the downstream \textit{tRNA}^{16S} gene, quantitation of 3'-ends present in the 16 S rRNA population demonstrates a scattering of termination frequency, with, for instance, the second most frequent termination point occurring at a template position 6 nucleotides downstream from the 5'-end of the \textit{tRNA}^{16S} gene.

Sequence Evolution of the Ribosomal RNA Region—In the sequence determination of the mouse mitochondrial rRNA region (3) it was noted that \textit{tRNA}_{\text{Val}}^{16S} was remarkably conventional in terms of its conserved bases and potential tertiary structure relative to \textit{tRNA}^{16S} and \textit{tRNA}^{16S}. Subsequent determination of the sequences of the remaining 19 tRNA genes (1) revealed that \textit{tRNA}_{\text{Leu}}^{Hr}, \textit{tRNA}_{\text{Val}}^{Cyto}, and \textit{tRNA}_{\text{His}}^{Omn} are the most orthodox tRNAs in this system in the sense that they retain nucleotides which are conserved in all other nonorganelle tRNAs which have been sequenced. It was suggested that the conservative nature of \textit{tRNA}_{\text{Leu}}^{Hr} could be rationalized if the sequence were under functional constraints in addition to a role in mitochondrial protein synthesis (9). Because a portion of this tRNA gene encodes the 3' terminus of a significant percentage of the 16 S rRNAs, and might additionally serve a transcription attenuation function, it can be argued that this gene cannot evolve as simply a tRNA coding sequence. These observations are consistent with a model in which the primordial mitochondrial genome had exclusively cytoplasmic-like tRNA genes, and that multiple functional demands on the \textit{tRNA}^{16S} gene sequences occurred early in evolution such that this gene has diverged more slowly than the majority of the other mitochondrial tRNA genes. It is interesting to note that because of the conservation of features of cytoplasmic tRNAs, the \textit{tRNA}^{16S} gene has also retained the internal control regions for regulation of RNA polymerase III-dependent transcription of nuclear tRNA genes, raising the possibility that a factor which modulates initiation of transcription of tRNA genes in the nucleus might be involved in control of \textit{rRNA} gene expression in the mitochondrion.

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