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The 3'-terminal sequence of the small subunit ribosomal RNA from hamster mitochondria

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ABSTRACT

The 220 3'-terminal nucleotides of the small ribosomal subunit RNA (13S) of hamster (BHK-21) cell mitochondria have been sequenced and the positions of post-transcriptionally methylated residues within this sequence have been established. Also, we have derived the secondary structure of the 3'-terminus of mitochondrial 13S rRNA by 1) searching nucleotide sequences of 13S rRNA, procaryotic 16S rRNA and eucaryotic 18S rRNA for common secondary structures and 2) using single-strand specific endonucleases to map secondary interactions in 13S rRNA.

The pyrimidine tract CCUCC in E. coli 16S rRNA, which participates in base-pairing with bacterial mRNA, is absent in mitochondrial 13S rRNA. We believe that the binding of mRNA to mammalian mitochondrial ribosomes is not mediated by a conventional Shine-Dalgarno interaction.

INTRODUCTION

The binding of mRNA to small ribosomal subunits of bacteria is facilitated by base pairing with the small ribosomal subunit (SSU[1]) RNA. This was first suggested by Shine and Dalgarno upon observing a 3-9 nucleotide complementarity between a polypyrimidine stretch near the 3'-terminus of E. coli 16S rRNA and polypurine sequences in the 5'-noncoding regions of procaryotic mRNAs (2). Further studies have confirmed this hypothesis and have assigned other active roles to the 3'-terminal region of 16S rRNA during protein synthesis (3-5). Sequence analysis of eucaryotic 18S rRNA reveals that a 45 nucleotide segment at the 3'-terminus is largely homologous to its bacterial counterpart except for the conspicuous absence of the sequence CCUCC in 18S rRNA (6-8). Since this pentanucleotide serves as the core of the mRNA binding sequence in E. coli 16S rRNA, it appears that the Shine-Dalgarno interaction is not a feature of eucaryotic protein synthesis. Indeed this may underscore a fundamental difference in the mechanisms by which eucaryotic and procaryotic ribosomes bind mRNA and select the proper initiation codon (9).

Mitochondrial ribosomes are commonly assumed to be functionally, and perhaps phylogenetically, related to bacterial ribosomes. That the initia-

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tion of mitochondrial protein synthesis resembles the procaryotic process - and consequently employs a Shine-Dalgarno interaction - is implied by three observations: 1) mitochondrial ribosomes display a procaryotic spectrum of antibiotic sensitivity (10), 2) mitochondrial mRNAs are unmethylated and uncapped (11,12) and 3) mitochondrial initiator tRNA is formylated (13). To begin to elucidate the role of rRNA during mitochondrial protein synthesis, we have sequenced the 3'-terminal 220 nucleotides of 13S rRNA, the small ribosomal subunit RNA of mammalian mitochondria. By incorporating previous data on the post-transcriptional modifications of 13S rRNA (14; also, Baer & Dubin, in preparation) we are able to present the complete primary structure of this RNA region. Suprisingly, 13S rRNA lacks the mRNA-binding sequence described by Shine and Dalgarno (2). Secondary interactions between 3'-terminal nucleotides of 13S rRNA were uncovered with the use of endonucleases specific for single-stranded stretches of RNA. With these data we were able to derive the secondary structure of the 3'-terminus of 13S rRNA.

Some of these results were presented briefly at the Conference on the Organization and Expression of the Mitochondrial Genome, held at Bari, Italy in June, 1980 (15).

METHODS

Growth and labeling of cells and purification of 13S rRNA were as previously described (14,16,17). Mitochondrial 13S rRNA was labeled at its 3'-terminus with T4 RNA ligase (P-L Biochemicals) and [5'-^{32}P]pCp (New England Nuclear, 2000 ci/mmole) under conditions similar to those of England and Uhlenbeck (18,19). The reaction mixtures contained approximately 20 pmoles of 13S rRNA (7 µg), 30-60 pmoles of pCp, 100 pmoles of ATP and 7 units of RNA ligase. End-labeled 13S rRNA was repurified by electrophoresis on an acrylamide slab gel (3.5% acrylamide, 0.1% bisacrylamide; 1.5 mm x 13 mm) containing 7M urea.

The 3'-terminal nucleotide was identified by digesting 3' end-labeled 13S rRNA with T_2 RNase and fractionating the digest by electrophoresis on filter paper (20). Radioactive products were detected by autoradiography and quantitated by liquid scintillation counting. Mobility shift experiments were performed by hydrolyzing 3' end-labeled 13S rRNA with formamide (21) and fractionating the reaction products on a fingerprinting system (22,23).

For rapid RNA sequencing, the 3' end-labeled mitochondrial 13S rRNA was subjected to base-specific cleavage followed by electrophoresis on polyacrylamide gels. The enzymatic conditions of Donis-Keller *et al* (24) were used

for the RNase T_1, U_2 and A digestions. The reactions of the chemical sequencing procedure were performed as described by Peattie (25). The partial cleavage products of each reaction were fractionated by electrophoresis on thin (0.4 mm) 20%, 8% or 5% polyacrylamide (acrylamide:bisacrylamide; 20:1) gels containing 7M urea, and were detected by autoradiography with intensifying screens (26). The mapping of RNA secondary structure using S_1 nuclease and T_1 RNase has been described (27,28).

RESULTS

Isolation and sequence analysis of the 3'-terminal T_1-oligonucleotide of mitochondrial 13S rRNA

Using hamster cells (BHK-21) uniformly labeled with ^{32}P, we were able to identify the 3'-terminal T_1-oligonucleotide of 13S rRNA. Purified 13S rRNA was digested exhaustively with T_1 RNase and after 2-dimensional fractionation of the digest, oligonucleotide spots were assayed for the presence of Gp. The 3'-terminal T_1-oligonucleotide of an RNA is the only one which does not yield Gp upon digestion with T_2 RNase. A single spot, which we have designated 13S-T60 (cf Fig. 1, ref. 14; also Baer & Dubin, in preparation), present in molar yields, was found to lack Gp (Table 1). Two additional features of 13S-T60 suggest that it lacks a 3'-monoesterified phosphate, and thus represents the 3'-terminal oligonucleotide of 13S rRNA: 1) The position of 13S-T60 did not correlate with our determination (v.i.) of its base composition. It migrated exceptionally slowly in the electrophor-

Table 1. Sequence Determination of the 3'-Terminal T_1-oligonucleotide of Mitochondrial 13S rRNA

Alkali	T_2	P_1	VPDE	A	U_2
3Ap	3Ap	3pA	3pA	AACp	2Ap
2Cp	2Cp	2pC	2pC	ACp	(C,U)Ap
Up	Up	pU	pU	Up	

13S-T60 was digested to completion with each of the following enzymes: T_2 RNase (T_2), P_1 RNase (P_1), snake venom phosphodiesterase (VPDE), RNase A (A), and U_2 RNase (U_2). The mononucleotides released by alkali, T_2, P_1 or VPDE were separated by paper electrophoresis at pH 3.5 (20) and quantitated by scintillation counting. In all cases the values relative to Up or pU were within 10% of the given integers. U_2 digests and A digests were fractionated by electrophoresis on DEAE-paper (29) and the products were identified by 1) co-migration with oligonucleotide standards and 2) alkaline hydrolysis followed by electrophoretic analysis as above. During fractionation of the U_2 digest the product CpA_{OH} migrated toward cathode and thus was undetected.

etic dimension and rapidly in the chromatographic dimension, both of which can be attributed to the absence of a 3'-phosphate group. 2) 13S-T60 is degraded by venom phosphodiesterase (Table 1), an exonuclease whose activity is inhibited by 3'-phosphate groups.

In order to reveal its composition and sequence, 13S-T60 was hydrolyzed by alkali and by a variety of nucleases. Possible sequences, $AACUACA_{OH}$ and $ACUAACA_{OH}$, were derived by examination of the products of the enzyme digestions (Table 1). As will be seen below, the latter sequence is correct. We had previously proposed a somewhat different 3'-terminal sequence, $YUAUUA_{OH}$, on the basis of electrophoretic analysis of RNase A-released hydrazones after step-wise β-elimination of extreme terminal residues (17). The discrepancy may result from similar mobilities of the hydrazones released after elimination of the first residue (that of AAC vs. that of U) and after elimination of the second (that of AA vs. that of AU). The hydrazones released after zero, 3 and 4 eliminations would be the same for both sequences.

Sequence analysis of 3' end-labeled mitochondrial 13S rRNA

To apply rapid sequencing procedures, we prepared 3' end-labeled mitochondrial 13S rRNA. 13S rRNA was isolated from unlabeled cells, and after reaction with [5'-^{32}P]pCp and T4 RNA ligase, was repurified by polyacrylamide gel electrophoresis. Before sequencing, an aliquot of the 3' end-labeled 13S rRNA was digested with T_2 RNase and analyzed by electrophoresis on filter paper; >90% of the radioactivity migrated with the optical density marker for Ap.

A mobility shift experiment, shown in Figure 1, revealed the sequence of the fifteen 3'-terminal nucleotides. For further sequence analysis, 3' end-labeled 13S rRNA was partially cleaved by a series of base-specific enzymatic (21,24) or chemical (25) reactions and the resulting RNA fragments were fractionated on sequencing gels (26). The enzymatic procedure (Fig. 2A) revealed the sequence of guanines, adenines and pyrimidines. The chemical procedure (Fig. 2B) provided the same information and, in addition, discriminated between cytosine and uracil residues. We were able to discern unequivocally the sequence of the 3'-terminal 220 nucleotides (Fig. 3).

Previously we had determined sequences of the methylated oligonucleotides of mitochondrial rRNAs (14; also, Baer & Dubin, in preparation). Two such methylated sequences, $YG{\cdot}m^4C(C,m^5C)G$ and $YGG{\cdot}m^6_2A{\cdot}m^6_2A{\cdot}AGU$, accounting for 4 of the 5 methylated residues of 13S rRNA, were localized within the 3'-terminal sequence (Fig. 3). This positioning of the methylated nucleotides is supported by comparisons with homologous methylated residues in

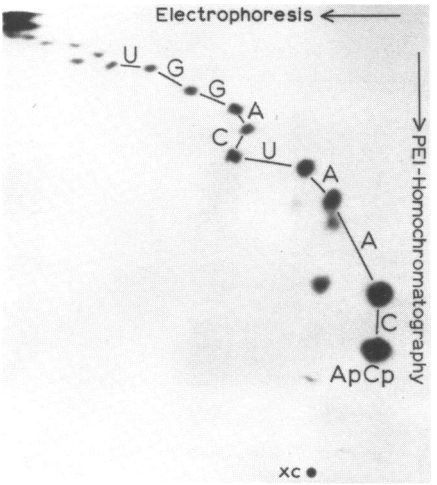

Figure 1. Mobility shift experiment. 3' End-labeled 13S rRNA was partially digested with formamide and the products were fractionated by fingerprinting. The faint "shadows" of some of the major spots can be attributed to the fact that the RNA is actually labeled at the penultimate phosphate (cf ref. 30).

bacterial 16S rRNA and eucaryotic 18S rRNA. Also, the effect of the m_2^6A modification during the sequencing reactions is obvious. Residues of m_2^6A are resistant to U_2 RNase (6), as can be seen in Figure 2A. In the "A>G" chemical reaction, m_2^6A residues were poorly cleaved and thus appear like G bands (Fig. 2B); however, unlike true guanines, m_2^6A residues were not sensitive to the "G" chemical reaction. The modified cytosines, m^4C and m^5C, did not show anomalous sensitivities to the sequencing reactions.

Secondary structure analysis of 3' end-labeled mitochondrial 13S rRNA

In Figure 3 the 3'-terminal sequence of mitochondrial 13S rRNA has been aligned with homologous sequences from E. coli 16S rRNA (31,32) and B. mori 18S rRNA (8) using as guides the primary sequence and modified residues of 16S rRNA conserved regions nos. 1 and 2 (35; also see DISCUSSION). In order to maintain molecular conformations which are necessary for ribosome function, it seems probable that rRNAs have not only retained certain primary sequences but have also preserved common secondary structures. Therefore, we searched, within the conserved regions, for local secondary structures that might be common to 13S, 16S and 18S rRNAs. We have found that nucleotides of sections 1b, 2c and 2e (Fig. 3) can form conserved hairpins, which we designate I, II and III, respectively (15; also, Fig. 5). Corresponding hairpins

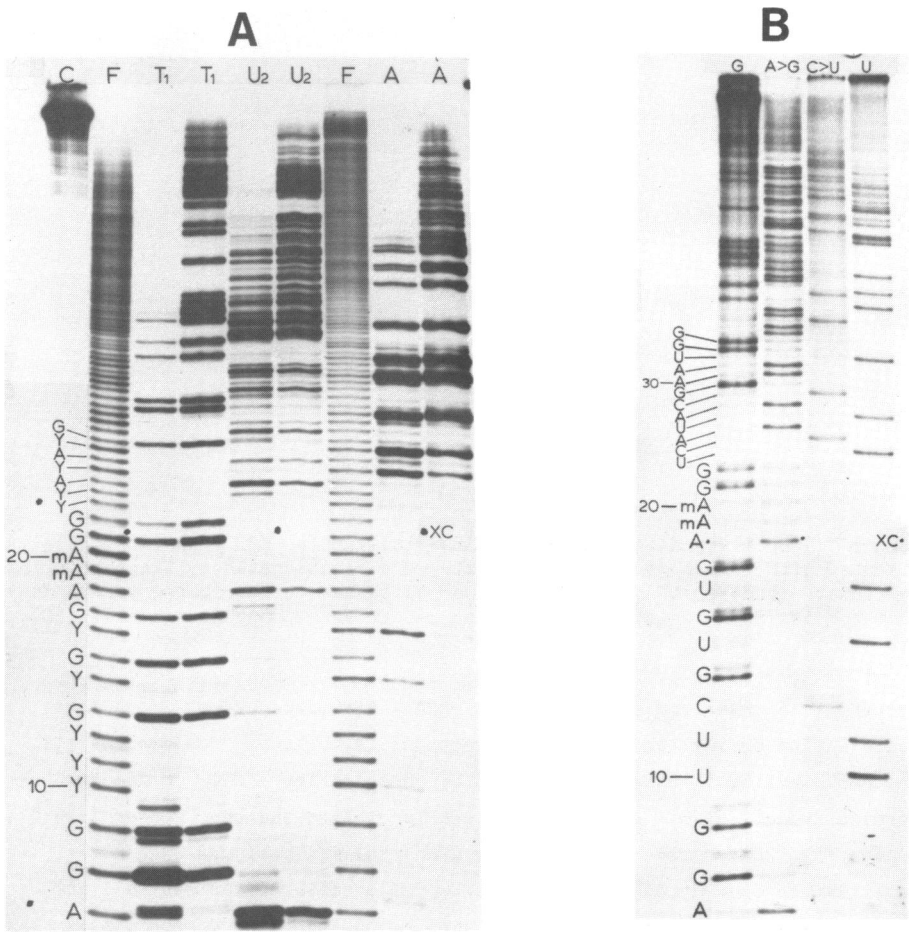

Figure 2. Sequencing gels of mitochondrial 13S rRNA. (A) Partial enzymatic digests of 3' end-labeled 13S rRNA were fractionated on a 20% polyacrylamide gel: Control (C), Formamide (F), T_1 RNase (T_1), U_2 RNase (U_2), and RNase A (A). The dye marker, xylene cyanol, is designated "XC". (B) Partial chemical digests of 3' end-labeled 13S rRNA were fractionated on a 20% polyacrylamide gel. The lane headings refer to the base specificities of the chemically-induced cleavages. Here, as elsewhere in the paper, 13S rRNA nucleotides are numbered counting from the 3'-end.

can also be constructed from the recently published sequences of chloroplast 16S rRNA (40) and yeast 18S rRNA (41). The existence of these hairpins in free rRNA was tested by digesting 3' end-labeled 13S rRNA, under nondenaturing conditions, with S_1 nuclease (27) or T_1 RNase (28) and fractionating the

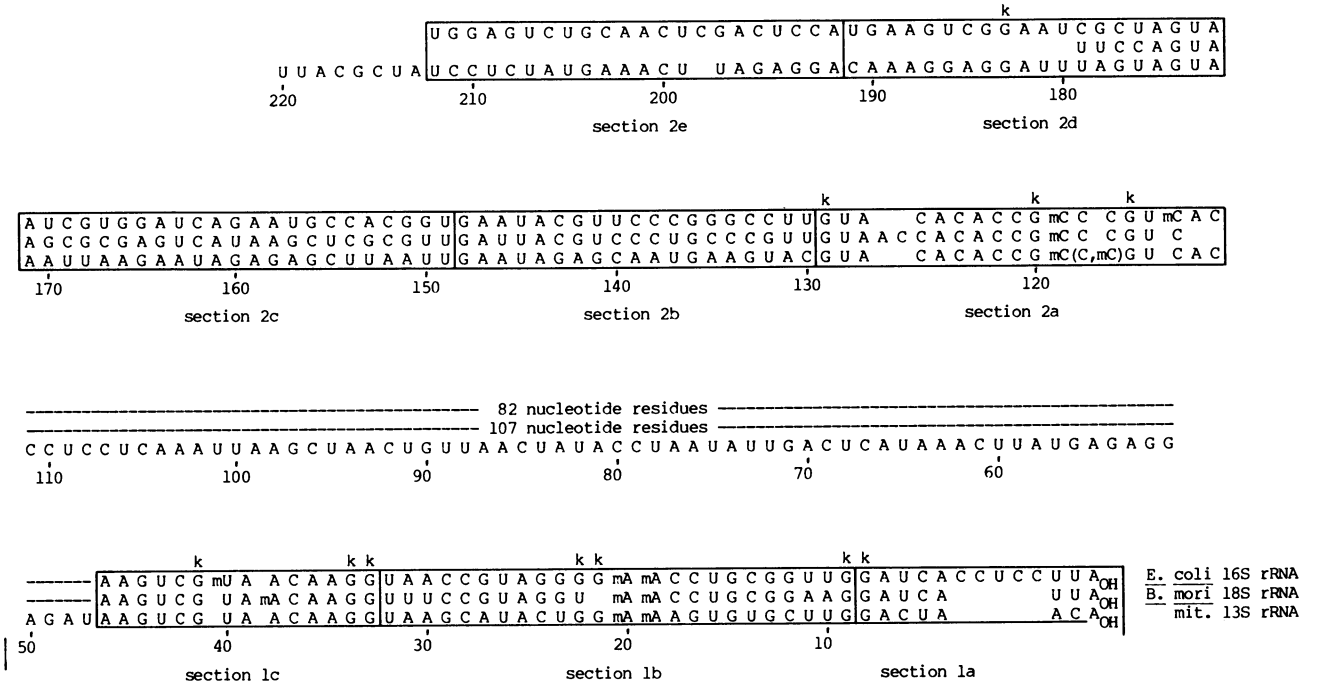

Figure 3. 3'-Terminal sequence of mitochondrial 13S rRNA aligned with homologous sequences from E. coli 16S and B. mori 18S rRNAs (8,31,32). Nucleotide numbering refers to 13S rRNA. Kethoxal-sensitive residues of 16S rRNA are indicated by "k" (33,34). Conserved regions 1 and 2 correspond to the designations of Woese et al for procaryotic 16S rRNA (35). Each conserved region has been subdivided into "sections" in order to facilitate discussion of secondary structure. Nucleotides of sections 1b, 2c and 2e can form hairpins ^{4}I, II and III, respectively, as shown in Figure 5. The identities of the modified nucleosides of 13S rRNA are m^4C, m^5C, and $m_2^6Am_2^6A$ (14,20). The modified nucleosides of 18S rRNA are presumed to be Cm, m^6A, and $m_2^6Am_2^6A$ (corresponding to oligonucleotides T8, T34 and T30, respectively, of Maden and Reeder [36], and oligonucleotides 50, 78 and 42, respectively, of Choi and Busch [37]). The modified nucleosides of E. coli 16S rRNA are, from 5' to 3', m^4C m^5C, m^3U and $m_2^6Am_2^6A$ (20,32,38,39).

products on sequencing gels. In Figure 4, it can be seen that nucleotides comprising the loops of hairpins I and III are cut by T_1 RNase (i.e., G_{21}, G_{22}, G_{204}) whereas the stem nucleotides G_{13}, G_{15}, G_{17}, G_{29}, G_{193}, G_{194}, and G_{196} remain uncleaved. Likewise, these hairpins show a similar pattern of sensitivity to S_1 nuclease. The resistance of the loop of hairpin I to S_1 nuclease is probably due to the presence of guanines, which are poorly cleav-

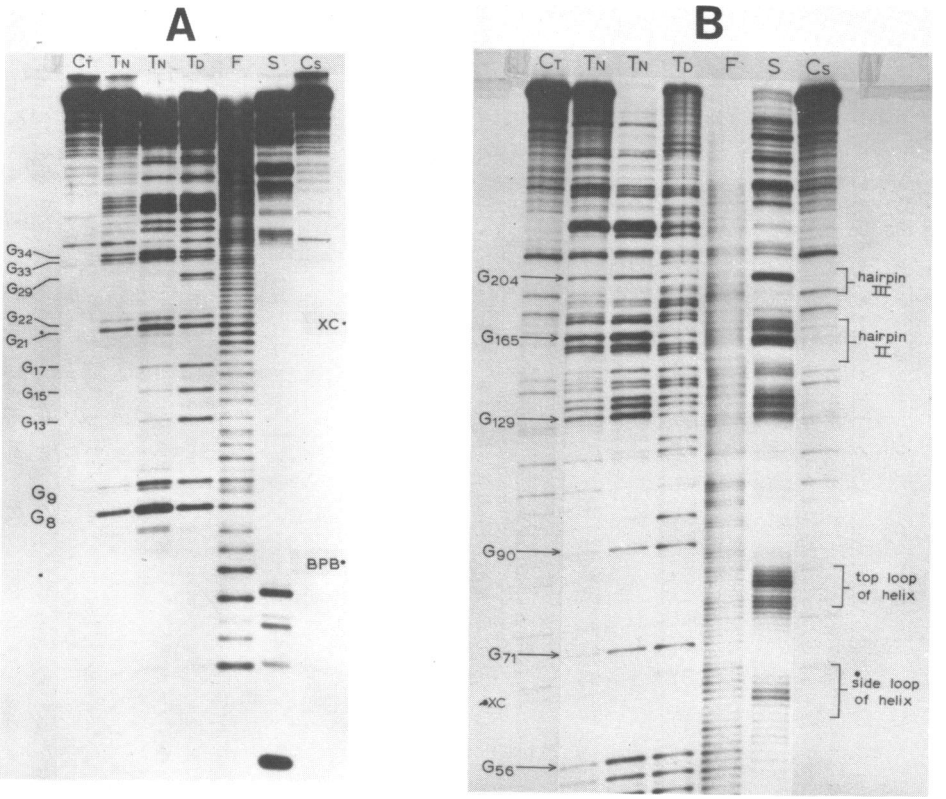

Figure 4. Secondary structure analysis of mitochondrial 13S rRNA. Partial endonuclease digests of 3' end-labeled 13S rRNA were fractionated on 20% (A) or 8% (B) polyacrylamide gels: Control for T_1 RNase digestion (C_T), T_1 RNase under nondenaturing conditions (T_N), T_1 RNase under denaturing conditions (T_D), formamide (F), S_1 nuclease under nondenaturing conditions (S), and control for S_1 nuclease digestion (C_S). Positions corresponding to several guanine residues are indicated on the left of each gel. The positions corresponding to hairpins II and III and the loops of the "poorly conserved helix" are denoted on the right of B. The dye markers bromphenol blue and xylene cyanol are designated by "BPB" and "XC", respectively.

ed by S_1 (42), and modified adenines. The evidence for the existence of hairpin II is less impressive; although the loop is extremely sensitive to endonuclease digestion, the 5'-stem nucleotides are also cleaved significantly. Perhaps, under these conditions, hairpin II is equilibrating with a competing secondary structure.

The primary sequence of nucleotides 47-111 of 13S rRNA (Fig. 3) is unrelated to sequences of E. coli 16S rRNA and B. mori 18S rRNA. We have not found secondary structures within this nonconserved region which are common to all SSU RNAs. However, Ross and Brimacombe have shown that nucleotides of the 16S rRNA nonconserved region can form a long, incompletely double-stranded helix (43). Similarly, the nonconserved region of 13S rRNA can also form a long helix (designated as the "poorly conserved helix" in Figure 5) which, although generally similar, differs from the 16S rRNA helix considerably in detail. Within the putative 13S rRNA helix there are two extensive single-stranded stretches (the "3'-side loop", nucleotides 61-67; and the "top loop", nucleotides 79-86), both of which are sensitive to cleavage by S_1 nuclease (Fig. 4B).

Although our structural analysis of 13S rRNA only considers local secondary interactions, 3'-terminal nucleotides may also base-pair with distant residues of the molecule. Indeed, Ehresmann et al have implicated 16S rRNA nucleotides of section 2b in a long-range interaction with a complementary sequence located approximately 450 residues away (44).

DISCUSSION

Comparative aspects of the 3'-terminal segment of SSU RNA

The topological arrangement of rRNA sequences within the ribosome has been explored most extensively in work with the small ribosomal subunit of prokaryotes. Woese et al have compared "T_1-oligomer" catalogues of 16S rRNA from a variety of bacteria (35). On correlating these data with the primary sequence of E. coli 16S rRNA (31,32) it became apparent that those oligomers that tend to be conserved evolutionarily are located in several discrete regions of the molecule, and that these regions include the methylated residues (38,39), as well as the residues occupying the surface of the ribosomal subunit by the criteria of preferential kethoxal or nuclease sensitivity (33-35,45). It is clear from a perusal of Figure 3 that the 220 nucleotide sequence of mitochondrial 13S rRNA which we present encompasses the two conserved regions of 16S rRNA nearest the 3'-terminus (nos. "1" and "2"). Our ability to align long stretches of the 13S rRNA sequence with these conserved

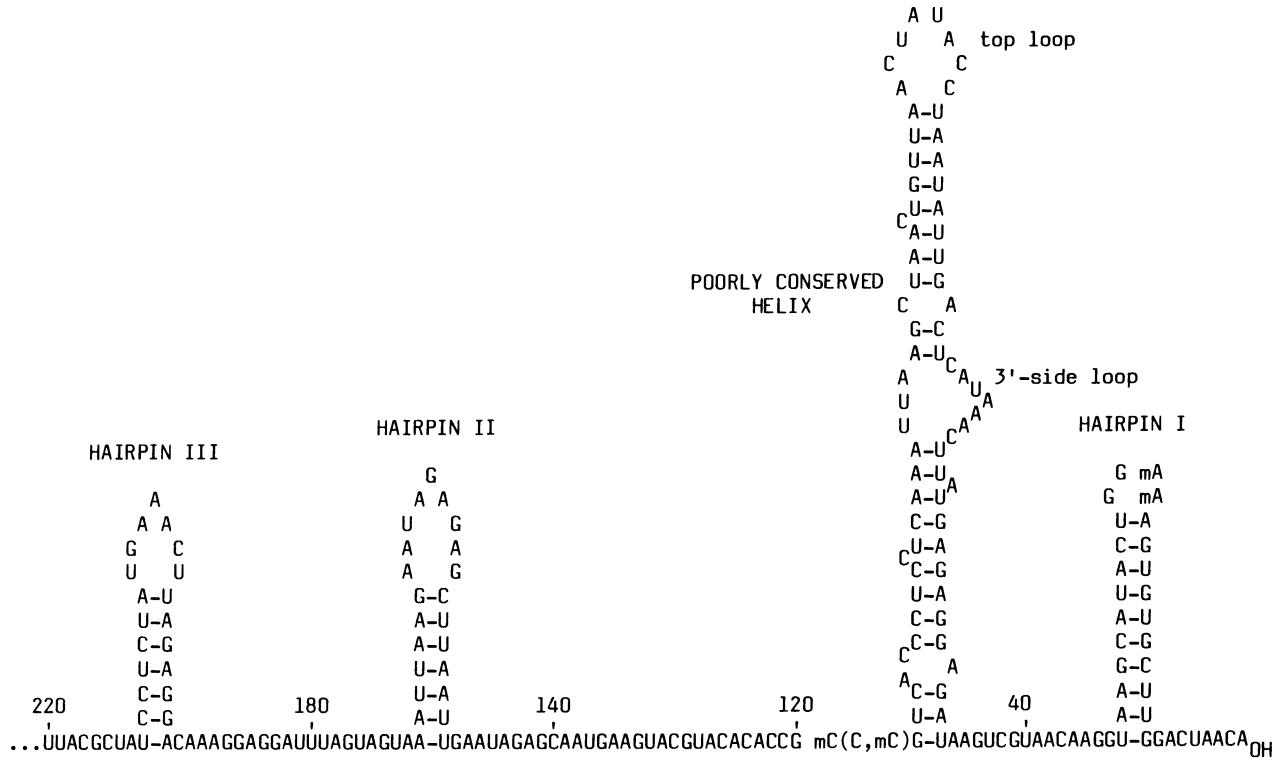

Figure 5. Proposed secondary structure at the 3'-end of mitochondrial 13S rRNA.

regions and with corresponding regions of 18S rRNA (Fig. 3) indicates that such conservation extends to mitochondrial and eucaryotic SSU RNAs. Perhaps even more striking is the apparent conservation of secondary structure in these regions, in view of the relative divergence of primary sequence within the stems of the proposed hairpins (Figs. 3, 5). Of course this is precisely as expected if the helices per se (and hence the hairpin configurations) have been selected for; since the base moieties of helical stretches are largely inaccessible, presumably these bases may mutate so long as compensating mutations permit maintenance of the helix. These results indicate universal roles for primary and secondary structural features of the conserved 3'-regions of SSU RNA; and taken together with the topological inferences on the E. coli 30S subunit noted above, they suggest that these roles involve interaction with the large ribosomal subunit and/or with extra-ribosomal ligands.

During preparation of this manuscript we became aware of recent studies by Eperon et al (46) and Van Etten et al (47) in which primary sequences of human and mouse mitochondrial rRNA genes are determined. The sequences of the 3'-segments of the two rodent mitochondrial SSU RNAs are >85% homologous; satisfyingly, the differences that do occur are concentrated in what we refer to here as the nonconserved region. Also, complete secondary structure models for E. coli 16S rRNA were recently presented by Woese et al (48) and Glotz and Brimacombe (49). The proposed 3'-structures of 16S rRNA resemble that of mitochondrial 13S rRNA (Fig. 5) in that they contain the conserved hairpins I, II and III as well as the "poorly conserved helix".

Methylated residues of mitochondrial 13S rRNA and implications concerning the corresponding methylases

The clustering of modified residues in the 3'-terminal region of mitochondrial 13S rRNA is in agreement with findings for 16S rRNA (32) and 18S rRNA (36). The definition of primary sequences and secondary structures around the methylated residues of 13S rRNA allows meaningful speculation on the biology of the corresponding methylases. Due to the limited coding potential of the mammalian mitochondrial genome (10), it is likely that mitochondrial RNA methylases are synthesized on cytoplasmic ribosomes and are subsequently transported into the organelle. We have reported that the methylated cytosine nucleosides of 13S rRNA, m^4C and m^5C, are absent from cytoplasmic 18S rRNA (20). Yet the strict sequence and structural homologies between conserved regions nos. 2 of 13S and 18S rRNAs, and the fact that region 2 remains exposed in the intact subunit (33-35,45), would suggest that the mitochondrial enzymes responsible for the production of m^4C and m^5C

would also be capable of modifying cytoplasmic 18S rRNA. Therefore the synthesis and transport of these mitochondrial cytosine methylases are probably tightly coupled so as to maintain compartmentalization between the enzymes and the cytoplasmic ribosomes. On the other hand, the enzymes responsible for the production of mitochondrial and cytoplasmic m_2^6A could conceivably be the same, recognizing the conserved structure of hairpin I (Fig. 5) and methylating the loop nucleotides accordingly. It is interesting in this regard that although most post-transcriptional modifications of 18S rRNA occur in the nucleus, the formation of m_2^6A occurs in the cytoplasm (50).

Mitochondrial 13S rRNA lacks mRNA-binding sequences

Perhaps the most surprising feature of the 3'-terminal sequence of mitochondrial 13S rRNA is the absence of the pyrimidine pentanucleotide CCUCC, which participates in base-pairing with mRNA in *E. coli* (2,51). This circumstance suggests that the binding of mRNA to mammalian mitochondrial ribosomes is not mediated by a conventional Shine-Dalgarno interaction. In order to evaluate this hypothesis, it would be useful to examine nucleotide sequences in 5'-noncoding regions of mammalian mitochondrial mRNAs. Although no such mRNA sequence data are available, Barrell *et al* have shown that the initiator codon of the human gene for cytochrome oxidase subunit II (COII) is immediately preceded by a gene coding for $tRNA^{Asp}$ (52). If RNA splicing does *not* occur in animal mitochondria (as appears to be the case [53]), then any 5'-noncoding region of COII mRNA that occurs must contain $tRNA^{Asp}$ sequences. We have located short sequences within this putative 5'-noncoding region of COII mRNA which could base-pair with 13S rRNA. However, none of these complementarities incorporate the parameters of a conventional Shine-Dalgarno interaction - namely, base-pairing between a mRNA sequence centered 8-12 nucleotides 5' of the initiator codon and a rRNA sequence located on the 3'-side of the m_2^6A-containing hairpin. Of course, it is possible that transcripts of the $tRNA^{Asp}$ gene are rapidly and quantitatively converted to mature tRNA, thus leaving the COII message without a 5'-noncoding region. This situation would not be entirely unprecedented; in lambda p_{RE} repressor mRNA, the initiating triplet, AUG, comprises the three 5'-terminal nucleotides (54). In any case, we believe that the initiation of mitochondrial protein synthesis, although sharing several features with the analogous process in bacteria (see INTRODUCTION), does not involve a conventional Shine-Dalgarno interaction.

The use of a Shine-Dalgarno interaction has two important consequences for protein synthesis on bacterial ribosomes. First, the Shine-Dalgarno interaction allows ribosomes to identify initiation sites anywhere along the

mRNA sequence. Therefore procaryotic ribosomes, unlike eucaryotic ribosomes, can translate polycistronic messages (9,55). Secondly, the Shine-Dalgarno interaction provides a potential mechanism for differential mRNA expression; those messages which exhibit a greater complementarity to 16S rRNA - other things being equal - will be translated more efficiently (51,56). It is interesting to note that mitochondrial ribosomes may not be required to serve either of these two functions. The rough equivalence between the number of mitochondrial-coded poly(A)$^+$ RNA species and the number of mitochondrial-coded polypeptides (57) suggests that mitochondrial messages may be, as a class, monocistronic. Also, mitochondrial ribosomes may not need to translate different messages at different efficiencies; the major products of mitochondrial protein synthesis, the respiratory polypeptides, are probably required in approximately equimolar quantities by the cell.

The origin of mitochondria

Because of the uncertain phylogenetic status of mitochondria, contending evolutionary hypotheses are constantly reappraised as new data emerge. Our work with mitochondrial 13S rRNA does not serve to clarify the issue. The most popular theory, in which mitochondria represent vestiges of an ancient bacterial endosymbiont, derives some support from the fact that, within conserved regions nos. 1 and 2, mitochondrial 13S rRNA does share a slightly greater homology with its procaryotic counterpart than with its eucaryotic counterpart. However, by this criterion, 16S and 18S rRNAs are more closely related to each other than either is to mitochondrial 13S rRNA. Furthermore our results suggests that the strategy of mRNA-binding by mitochondrial ribosomes may be unique. On one hand, mitochondrial mRNAs, unlike eucaryotic messages, are unmethylated and uncapped (10,11). On the other hand, mitochondrial ribosomes, unlike procaryotic ribosomes, probably do not make use of a conventional Shine-Dalgarno interaction during the initial binding of mRNA.

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