Paramecium Mitochondrial Genes

I. SMALL SUBUNIT rRNA GENE SEQUENCE AND MICROEVOLUTION

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The sequences of the small subunit mitochondrial rRNA genes from two divergent species of Paramecium (primaurella and tetraurelia) were determined. The gene lies near the center of the linear mitochondrial genome, on the same strand as are all other currently identified genes. The sequences generally resemble their counterparts found in cytoplasmic, procaricotic, and other mitochondrial sources. The rDNA gene boundaries were located by nuclease S1 protection. Small subunit rDNA spans about 1680 nucleotides, including an extraneous 83-base pair sequence very near the 3′ end which is unique to Paramecium mitochondria. This "insert" occurs at the apex of the highly variable in length penultimate helix, according to proposed models for small subunit rRNA secondary structure. A discontinuity occurs in isolated rRNA near the start of the insert, resulting in a stable 13 S RNA species and a small segment containing the remaining 3′ portion of the gene.

The overall rRNA gene sequence was 94% conserved between the two species, and the nucleotide differences consisted of 53% transitions, 37% transversions, and 9% insertions plus deletions. These substitutions were somewhat clustered, and the two most divergent regions coincided with the gene boundaries. The sequence was aligned with Escherichia coli 16 S rRNA for direct comparison of sequence and structure.

In recent years, rRNA sequences from a wide variety of organisms have been determined (1-13). The increasing availability of such sequences has allowed the construction of tentative models for both evolutionary pathways as well as ribosome structure (14-20). In particular, the structural evolution of small subunit rRNAs has been of recent interest and particularly divergent. Sequence comparisons between species should produce the greatest number of base changes. Such a comparison should therefore allow us to 1) determine the extent of genetic drift within Paramecium mitochondrial genes, and 2) draw stronger conclusions concerning evolution and/or relative functional importance of key gene regions.

In previous work, we aligned DNA restriction fragments from these two species and found that the rRNA genes were located similarly in both species (27, 28). It was not known, however, to what degree this similarity in gene organization extended. Presently, we find both here and in the accompanying paper (40) that although only 50% of mitochondrial DNA from species 1 and 4 cross-hybridizes even under non-stringent conditions (27), the overall gene order is retained in all of the species. Consequently, although small (2 bases or less) deletions and insertions do occur, particularly within intergenic regions, the evolution of the entire genome can be considered with respect to substitutions on a base by base level.

In addition, we have used nuclease S1 protection to determine the approximate gene boundaries of rDNA. We examined these end regions for conserved sequences that could possibly be involved in transcriptional regulation. Although no clear correlations of this nature were revealed, we did find.
also in the accompanying paper surprisingly, that small subunit rDNA contains at least one region near the 3' end which appears to be removed from isolated small subunit (13S) rRNA. A shorter but analogous “deletable segment” was found within Paramecium mitochondrial large subunit rDNA. These results are discussed in relation to proposed secondary structures for rRNA here and also in the accompanying paper (40).

MATERIALS AND METHODS

Paramecium primaurelia (or species 1) stock 513 and Paramecium tetraurelia (or species 4) stock 51 were obtained from the Edinburgh stocks and were grown in bacterial cultures of Scotch grass. Axenic strains of P. tetraurelia stock 51, used for most RNA analyses, were obtained from the laboratory of Edna Kaneshiro (University of Cincinnati, Cincinnati, OH), and were grown in Soló’s axenic medium (29).

DNA isolation, RNA extraction, and RNA gel electrophoresis were performed as described previously (27, 35). RNA analysis was done by Northern blotting of 0.75 mM methyl mercuric hydroxide-agarose gels onto nitrocellulose filters using the Thomas method (30) with minor modifications (31). Specific DNA restriction fragments were obtained using previously isolated clones (27, 28). DNA fragments were sequenced using the Maxam and Gilbert method (32).

Nuclease S1 protection mapping was performed as described by Berk and Sharp (33), and the precipitated reaction products were examined on lanes adjacent to sequence ladders for precise length determination. Optimal results were obtained using a 4-h hybridization and a 45-min digestion at 37 °C in the presence of 5000 Boehringer Mannheim units of enzyme. Mitochondrial RNA for nuclease P1 protection experiments usually was obtained from species 4 stock 51 axenically grown cells; however, mitochondrial RNA from bacterized cultures of species 1 gave equivalent results. Gene 5’ ends were located using DNA probes which were 5’ end-labeled with polynucleotide kinase (Bethesda Research Laboratories). For 3’ ends, 3’-labeled DNA probes were made by DNA polymerase fill-in with the appropriate [32P]dNTP(s) of 5’-protruding ends left after restriction enzyme digestion. All enzymes were obtained from Bethesda Research Laboratories, except DNA polymerase I Klenow fragment and calf alkaline phosphatase (Boehringer Mannheim).

RESULTS AND DISCUSSION

Small Subunit rDNA Localization—Paramecium mitochondrial rDNA obtained from cultures of species 4 (axenic) and species 1 (grown on Klebsiella) was examined by electrophoresis on both methyl mercury-agarose (Fig. 1) and urea-acrylamide (not shown) gels. rRNAs of Paramecium nuclei and mitochondria and of the food source bacteria are present and clearly distinguishable. When these RNAs were Northern-blotted onto nitrocellulose and hybridized with appropriate DNA restriction fragment probes, transcripts of the small subunit rRNA gene could be identified. Note (Fig. 1, lane C) that one band was predominant, and that no larger sized precursor nor small sized degradation products were readily visible. Paramecium small subunit rRNA (previously determined to be 13S [27, 28]) migrated significantly ahead of E. coli 16 S RNA (1542 bases (1)). Based on the observed mobilities of the procaryotic and known Paramecium rRNAs present, 13S rRNA exhibited an empirical molecular size of ~1400 nucleotides.

The small subunit rRNA gene region within the genome was more clearly defined using smaller DNA fragments as hybridization probes (Fig. 1, lower). The rDNA was found to be contained within HindIII fragments 7 and 17 (species 1) and 8 and 3B (species 4) with respect to previous restriction maps (27, 28). The genes were found to be located near the center of the genome in both species and to be transcribed in the same direction in which DNA replication proceeds.

Small Subunit rDNA Sequence—In Fig. 2, the complete rDNA sequences for species 1 and 4 are shown. Most regions of the sequence have been covered from at least two different label points (both strands where possible); however, some regions could be covered from only one label point due to lack of convenient restriction sites within the sequence. In the latter regions, the sequence integrity is supported by homology between the two species. All nucleotide differences between the two species have been rechecked. The rRNA genes of both species are clearly homologous, allowing unambiguous alignment in all but a few short regions. The entire gene region contains 107 base differences between the two species (6.4% divergence), consisting of 53% transitions, 37% transversions, and 9% insertions/deletions.
Fig. 2. Small subunit rDNA sequence. The sequence of small subunit rRNA and flanking regions was determined using Maxam and Gilbert (32) chemistry. The identity strand sequences for _P. aeruginosa_ species 1 and 4 are shown mutually aligned (rows 2 and 3, respectively), and the nucleotide differences are shown in row 1. Regions containing the putative gene boundaries according to nuclease mapping data (see below).

A proposed alignment with _E. coli_ 16 S rRNA (1, 18) is also presented in Fig. 2. The alignment was derived empirically using computer-assisted visual alignment of primary structure and by taking into account the constraints imposed by proposed models for small subunit rRNAs secondary structure (14–18). We have used _E. coli_ rDNAs as alignment models for two reasons. First, the _E. coli_ sequences more than any other sequences are spread throughout the genome, and there are three intragenic clusters containing a slightly higher rate of base substitution (bases 246–316, 519–556, and 1539–1588 in Fig. 2, species 1 numbers). These clusters fall within regions of little or no homology to _E. coli_, suggesting that primary structure within these regions is less constrained evolutionarily. The two most divergent regions (bases 1–50 and 1694–1717, species 1 numbers) lie immediately adjacent and include both ends of rDNA. These "hypervariable" blocks fall outside the boundaries of the _E. coli_ 16 S rRNA sequence, but appear to contain the true ends of _Paramaecium_ mitochondrial rDNA based on nuclease S1 mapping data (see below).

Overall A + T content is 63%, identical to genomic DNA. In general, the base changes are spread throughout the genome nonrandomly. There are three intragenic clusters containing a slightly higher rate of base substitution (bases 246–316, 519–556, and 1539–1588 in Fig. 2, species 1 numbers). These clusters fall within regions of little or no homology to _E. coli_, suggesting that primary structure within these regions is less constrained evolutionarily. The two most divergent regions (bases 1–50 and 1694–1717, species 1 numbers) lie immediately adjacent and include both ends of rDNA. These "hypervariable" blocks fall outside the boundaries of the _E. coli_ 16 S rRNA sequence, but appear to contain the true ends of _Paramaecium_ mitochondrial rDNA based on nuclease S1 mapping data (see below).
species 4, S1 data not shown). Curiously, the protected bands were obtained with species 1. This result suggested small protected to full length (at least 2000 bases). Similar results appeared atypically faint, and most probe molecules were adjacent to sequencing chemistries of the same fragment.

In determining the small subunit rDNA 3′ end, we suspected that the end would parallel the 3′ end of the E. coli sequence. We prepared probes accordingly, using the fragment extending rightward from the XbaI site at base 1485 in species 4. Indeed, S1 protection bands were observed corresponding to nucleotides 1706-1713. Again, the protected bands appeared faint, similar to the results at the 5′ end, except that no full length protection was observed.

Unfortunately, these nuclease S1 data did not allow precise determinations of the rDNA ends; accurate placement will ultimately require sequence analysis of the rRNA itself. However, using tentative assignments of base 30 (start) and 1710 (stop, species 1 numbers), small subunit rDNA spans about 1680 bases. This length was significantly greater than expected, based upon electrophoretic mobilities of the RNA observed (~1400 nucleotides; Fig. 1). Because of this discrepancy, we suspected that a segment of the nascent small subunit transcript was removed and that 13 S rRNA was the major cleavage product. The most likely cleavage site was at or within the 83-base "insert" mentioned above, lying within the gene near the 3′ end. Therefore, we prepared a 3′ probe as near as possible upstream of this region. The only convenient restriction site available was the HindIII site at base 1086, and the S1-protected bands are shown in Fig. 3. Indeed, S1 protection bands were observed both at the gene terminus and the 3′ terminal region of Paramecium small subunit rRNA (e.g. Refs. 14-18). We propose in Fig. 4 a secondary structure model for the 3′-terminal region of Paramecium small subunit rRNA based on these structures and our results. The Paramecium insert is contained within the penultimate stem and loop structure of the E. coli sequence (helix P in Fig. 4, bases 1409–1445/1457–1491 of the E. coli structure (1, 18)). The insert is notably devoid of possible helices and therefore probably exists as a single stranded region in vivo. We have therefore included these and neighboring nucleotides of undetermined structure (bases 1487–1618) as a block. Note that the insert other much lighter bands were visible (not labeled) which probably represent minor degradation products. This experiment was done under conditions of relative RNA excess in order to detect the presence of internal cleavage sites; therefore, the relative intensities of cleavage bands may not reflect the true amounts of the particular RNA species present. Also, note the major band representing full gene length rRNA (band T).

These results became more meaningful when compared to recent models for small subunit rRNA structure (e.g. Refs. 14–18). We propose in Fig. 4 a secondary structure model for the 3′-terminal region of Paramecium small subunit rRNA based on these structures and our results. The Paramecium insert is contained within the penultimate stem and loop structure of the E. coli sequence (helix P in Fig. 4, bases 1409–1445/1457–1491 of the E. coli structure (1, 18)). The insert is notably devoid of possible helices and therefore probably exists as a single stranded region in vivo. We have therefore included these and neighboring nucleotides of undetermined structure (bases 1487–1618) as a block. Note that the insert

FIG. 3. Small subunit rDNA 3′ end determination. An end-labeled DNA fragment extending rightward from the HindIII site at base 1086 (Fig. 2) was used as a nuclease S1 probe against total mitochondrial RNA (lane 4) and was loaded in lanes adjacent to XbaI (lane 2) and HhaI (lane 1) digests of the same fragment, shown undigested in lane 3. Sequencing lanes (C, T, A, and G) of a different DNA fragment were included to assist in length determination. Major S1 cleavage bands (solid arrows) appear at bases 1703–1713 (T) and 1505–1516 (T), and a minor band (open arrow) appears around base 1325. The insert relative to E. coli 16 S rRNA is represented by the vertical arrow.
of Fig. 3 representing full gene length RNA may have been published structure (15, 18) observed faint S1 bands may have resulted from hybridization end is located only -20 bases from the beginning of the excised protected by this minor species in addition to contiguous model. The region shown is the sequence small subunit rRNA present. The major $41-protected band in rat liver, stable RNAs resulting from cleavage at specific points within both the small subunit rRNA penultimate loop (38) and another loop near the 5' end (39) have been reported and were attributed similarly to increased susceptibility of these particular loops to endonucleolytic cleavage as a consequence of their topology within the ribosome. This could also be the case here, but the cleavage effect within the penultimate loop of *Paramecium* small subunit RNA is much more specific. First, the cleavage event in *Paramecium* is a complete (as opposed to partial) effect since all small subunit rRNAs detectable by Northern hybridization (Fig. 1) are 13 S in size. Cleavage at the insert at base 1325, in contrast, does appear to be a partial effect. Second, the entire penultimate stem of rat liver small subunit rRNA, like most other small subunit rRNAs, can base pair to form a helix (8). Only the first portion of this stem is possible in *Paramecium*, leaving a large single-stranded region. Third, the cleavage point coincides with a large inserted sequence relative to all other known small subunit rRNA sequences.

The separation of the 13 S and 3' terminal gene segments by such a large insert suggests the 3' segment may be in the process of evolving into a separate gene segment, similar to the gene fragmentation seen in large subunit rRNAs. An analogous insert falls within the large subunit rRNA gene of *Paramecium* mitochondria. The evolutionary implications of these inserts, along with small subunit and large subunit rRNA transcription and structure, are discussed further in the accompanying paper (40).

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Paramecium Small Subunit rRNA

Paramecium mitochondrial genes. I. Small subunit rRNA gene sequence and microevolution.
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