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Phylogenetic Origins of the Plant Mitochondrion Based on a Comparative Analysis of 5S Ribosomal RNA Sequences

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Summary. The complete nucleotide sequences of 5S ribosomal RNAs from *Rhodocyclus gelatinosa, Rhodobacter sphaeroides,* and *Pseudomonas cepacia* were determined. Comparisons of these 5S RNA sequences show that rather than being phylogenetically related to one another, the two photosynthetic bacterial 5S RNAs share more sequence and signature homology with the RNAs of two nonphotosynthetic strains. *Rhodobacter sphaeroides* is specifically related to *Paracoccus denitrificans* and *Rc. gelatinosa* is related to *Ps. cepacia.* These results support earlier 16S ribosomal RNA studies and add two important groups to the 5S RNA data base. Unique 5S RNA structural features previously found in *P. denitrificans* are present also in the 5S RNA of *Rb. sphaeroides*; these provide the basis for subdivisional signatures. The immediate consequence of our obtaining these new sequences is that we are able to clarify the phylogenetic origins of the plant mitochondrion. In particular, we find a close phylogenetic relationship between the plant mitochondria and members of the alpha subdivision of the purple photosynthetic bacteria, namely, *Rb. sphaeroides, P. denitrificans,* and *Rhodospirillum rubrum.*

Key words: Symbiosis — Plant mitochondria — 5S RNA — Evolution — Purple bacteria

Introduction

Macromolecular sequence analysis is now an established approach to gaining insight into evolutionary relationships among microorganisms (Woese et al. 1976; Almassy and Dickerson 1978; Gibson et al. 1979; Whatley 1981; Stackebrandt and Woese 1984). 5S RNA is particularly useful, because it is a highly conserved constituent of all ribosomes except those of certain mitochondria, is readily isolated, and is easily sequenced. Moreover, a data base of approximately 300 sequences currently exists against which new sequences can be compared.

Morphological and physiological data have been used to define the taxonomic classifications within the Rhodospirillaceae. However, studies of 16S RNA cataloging (Gibson et al. 1979) and cytochrome c2 (Almassy and Dickerson 1978) have challenged the use of these traditional criteria, and in particular have indicated that *Rhodobacter sphaeroides* (formerly, *Rhodopseudomonas sphaeroides*) and *Rhodocyclus gelatinosa* (formerly, *Rhodopseudomonas gelatinosa*) do not in fact belong in the same genus but rather are phylogenetically intermingled with a large number of nonphotosynthetic organisms (Gibson et al. 1979; Stackebrandt and Woese 1984). These studies form the basis for the proposed rearrangement of the species and genera of the purple nonsulfur bacteria by Imhoff et al. (1984). These authors have proposed the creation of two new genera; *Rhodobacter* and *Rhodophila.* This new scheme provides the practicing bacteriologist with deterministic criteria that are more consistent with the molecular data. In the larger view (Fox et al. 1980), it was found that the Rhodospirillaceae and their many nonphotosynthetic relatives are part of a major line of descent of the Gram-negative bacteria. This division of the eubacteria has three major sublines [alpha, beta, and gamma (Woese et al. 1984a,b)].

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Two of these, alpha and beta, contain members of the Rhodospirillaceae.

Herein we report the results of a phylogenetic comparative analysis of the purple photosynthetic bacteria based on 5S RNA sequence and signature data. These results confirm the earlier 16S ribosomal RNA studies and add for the first time the beta subdivision of the purple photosynthetic bacteria to the 5S RNA data base. In addition, we address the evolutionary relationships between the plant mitochondria and the purple photosynthetic bacteria by comparison of 5S RNA signatures. These signatures consist of the homologous helices III and IV and sequences proximal to these helices. Our data indicate that the plant mitochondria are likely to have descended from bacteria of the alpha subdivision of the purple photosynthetic bacteria (i.e., *Rhodobacter, Rhodospirillum,* and their nonphotosynthetic relatives) rather than from the genus *Rhodocyclus* and its relatives (beta subdivision) or from *Escherichia coli* and its relatives (gamma subdivision).

Experimental

Rhodocyclus gelatinosa was isolated from a salt marsh environment by K.R. and B.J. Clayton, who kindly provided it to us. *Rhodobacter sphaeroides* mutant strain 26 was kindly provided by Donald Berns of the Center for Laboratories and Research, State of New York, Department of Public Health, Albany, New York. *Pseudomonas cepacia* ATCC 17616 was obtained from the American Type Culture Collection.

The cells were suspended in TMA buffer (10 mM Tris·HCl [pH 7.8], 10 mM Mg $[OAc]_2$, 60 mM NH_4Cl, 5 mM β-mercaptoethanol) and sonicated at 0°C until microscopy revealed a majority of the cells to be disrupted. The cell extracts were deproteinated with phenol and the 5S RNAs were separated out by centrifugation and Sephadex G-100 chromatography. Final purification was by gel electrophoresis. The 3′ and 5′ ^{32}P labeling of 5S RNAs was as previously described (Donis-Keller et al. 1977; England and Uhlenbeck 1978).

The sequences from these organisms were determined by several overlapping methods: enzymatic digestions of ^{32}P-labeled 5S RNA run on sequencing gels (Donis-Keller et al. 1977), RNA sequencing gels using chemically treated 3′-labeled 5S RNA (Peattie 1979), mobility shift analysis (Silberklang et al. 1977), oligonucleotide cataloging (Uchida et al. 1974), and chromatography of ribonuclease P1-digested RNA for end determination (Andersen et al. 1982).

Results

The determined 5S RNA sequences of *Ps. cepacia, Rc. gelatinosa,* and *Rb. sphaeroides* are shown in Fig. 1. The 5S sequences from *Thermus thermophilus* (Komiya et al. 1983), *Rhodospirillum rubrum* (Newhouse et al. 1982), *Paracoccus denitrificans* (MacKay et al. 1982), *Triticum aestivum* mitochondria (Spencer et al. 1981), and *E. coli* (Brownlee et al. 1968) are included for comparison.

Table 1. Numbers of nucleotide differences among 5S RNAs from the purple nonsulfur bacteria and wheat mitochondria

	E. c.	*Rc. g.*	*P. d.*	*Rb. s.*	*T. a.*	*R. r.*	*Th. t.*
E. coli	—	—	—	—	—	—	—
Rc. gelatinosa	33	—	—	—	—	—	—
P. dentrificans	41	42	—	—	—	—	—
Rb. sphaeroides	41	40	8	—	—	—	—
T. aestivum mitochondria	68	68	60	60	—	—	—
R. rubrum	43	40	22	25	61	—	—
Th. thermophilus	35	41	45	43	64	39	—
Ps. cepacia	40	13	44	45	67	39	47

Table 2. Nucleotide differences in 5S RNA regions of high homology between 5S RNAs of purple bacteria and the wheat mitochondrion[a]

Species	No. of differences from *T. aetivum* mitochondrion RNA
Rb. sphaeroides	5
P. denitrificans	3
R. rubrum	5
Th. thermophilus	8
Rc. gelatinosa	16
Ps. cepacia	18
E. coli	15

[a] See sequences underscored in Fig. 1. These represent a total of 51 nucleotide positions

The 5S RNAs from the two members of the alpha subdivision, *Rb. sphaeroides* and *P. denitrificans,* differ in only eight positions (Table 1). In addition, these RNAs share unusual secondary structure signatures such as the helix II trimer mispairing (Fig. 2). They also share signatures with another member of this subdivision, *R. rubrum,* in that they all have a base-pair deletion in helix IV [Luehrsen and Fox (1981) helix nomenclature] (Fig. 3) and similar base pairing in helix III (Fig. 4). *Rhodobacter sphaeroides* and *P. denitrificans* 5S RNAs also display an intermediate amount of primary structure homology with *R. rubrum* 5S RNA (25 and 22 differences, respectively; Table 1).

The 5S RNAs of the two species of the beta subdivision, *Rc. gelatinosa* and *Ps. cepacia,* differ by 13 nucleotide substitutions. Both RNAs display base-pair deletions in helix IV (Fig. 3). That of *Ps. cepacia* has two deletions; that of *Rc. gelatinosa,* only one. In contrast, *Rc. gelatinosa* and *Rb. sphaeroides* 5S RNAs exhibit 40 nucleotide differences in their primary structure (Table 1), but share a base-pair deletion in helix IV (Fig. 3).

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E. coli                -UGCCUGGCGGCCGUAGCGCGGUG--GUCCCACCUGACCCCAUGCCGAACUCAG
Ps. cepacia            --GCCUGACGACCAUAGCGAGUCG--GUCCCACUCCUUCCCAUCCCGAACAGGA
Rc. gelatinosa         --GCCUGAUGACCAUAGCGAGGUG--GUCCCACUCCUUCCCAUCCCGAACAGGA
T. thermophilus        AUCCCCCGUGCCCAUAGCGGCGUG--GAACCACCCGUUCCCAUUCCGAACACGG
Rb. sphaeroides        ---UCUGGUGGCCAUAGCACGAGC--AAAACACCCGAUCCCAUCCCGAACUCGG
P. denitrificans       --GUCUGGUGGCCAAAGCACGAGC--AAAACACCCGAUCCCAUCCCGAACUCGG
R. rubrum              UGGCCUGGUGGUCAUUGCGGGCUC--GAAACACCCGAUCCCAUCCCGAACUCGG
T. aestivum mito.      -AACCGGGC-ACUACGGUGAGACGUGAAAACACCCGAUCCCAUUCCGACCUCGA

-----AAGUGAAACGCCGUAGCGCCGAUGGUAGUGUGGGGUCU-CCCCAUGCGAGAGUAGGGAACUGCCAGGCAU
-----CCGUGAAACGACUCUACGCCGAUGAUAGUGCGGAU--UC--CCEUGUGAAAGUAGGUAAUCGUCAGGCCU
-----UAGUGAAACGCCUUUGCGCCGAUGAUAGUGCGGGU--UC-CCCGUGUGAAAGUAGGACAUCGUCAGC---
-----AAGUGAAACGCGCCAGCGCCGAUGGUACUGGGCGGGCGACCGCCUGGGAGAGUAGGUCGGUGCGGGGCAU
-----UAGUUAAGUGCCGUCGCGCCAAUGGUACUGCGUCU-UAA-GACGUGGGAGAGUAGGUUGCCGCCAGACC-
-----CCGUUAAGUGCCGUAGCGCCAAUGGUACUGCGUCA-AAA-GACGUGGGAGAGUAGGUCACCGCCAGACC-
-----CCGUGAAAGAGCCCUGCGCCAAUGGUACUGCGUCU-UAA-GGCGUGGGAGAGUAGGUCGCCGCCAGGCCU
UAUAUAUGUGGAAUCGUCUUGCGCCAUAUGUACUG--AA-AUUG-UU--CGGGAGACAUGGUCAAAGCCCGGAA-
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Fig. 1. Primary structures of 5S RNAs from *E. coli* (Brownlee et al. 1968), *Ps. cepacia, Rc. gelatinosa, Th. thermophilus* (Komiya et al. 1983), *Rb. sphaeroides, P. denitrificans* (MacKay et al. 1982), *R. rubrum* (Newhouse et al. 1982), and *T. aestivum* mitochondria (Spencer et al. 1981). Alignment is in accordance with the consensus generalized structure of 5S RNA and the use of universal positions as guideposts (Delihas et al. 1984). Dashes indicate alignment gaps

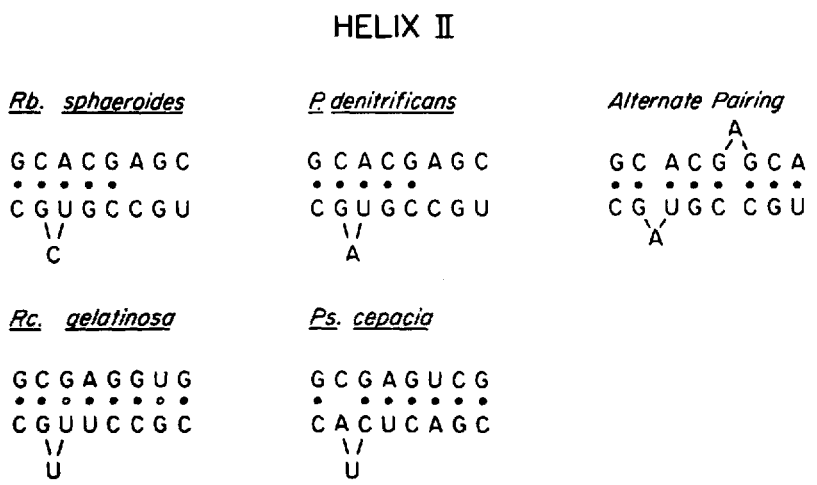

Fig. 2. Depiction of helix II trimer mispairing in 5S RNAs of *Rb. sphaeroides* and *P. denitrificans*. An alternate pairing scheme is also shown. *Rc. gelatinosa* and *Ps. cepacia* 5S RNAs are more representative of the generalized structure in this region of the molecule

The 5S RNA of the *T. aestivum* mitochondria shows its closest overall sequence homology to that of the organisms of the alpha subdivision of the purple bacteria (Table 1). In addition, selected regions that are highly homologous between the 5S RNAs of the purple photosynthetic bacteria and the wheat mitochondria have been compared (Table 2). These are underlined in Fig. 1. These selected regions show a closer homology of the wheat mitochondrial 5S RNA to the 5S RNAs of the alpha-subdivision bacteria than to the 5S RNAs of either *Rc. gelatinosa* and its relatives (beta subdivision) or *E. coli* (gamma subdivision). The comparable regions of the 5S RNA of *Th. thermophilus* show a homology to the wheat mitochondrial 5S RNA that is approximately intermediate between those of the alpha and beta subdivisions. The mitochondrial 5S RNA also contains the alpha-subdivision helix III signatures (Fig. 4), as well as similarities to the helix IV deletion signatures (Fig. 3). Thus there are close homologies between the wheat mitochondrial and alpha-subdivisional 5S RNAs in the regions of the molecule that encompass helices III and IV and sequences proximal to these helices.

A sequence comparison between the plant mitochondrial 5S RNA and the 5S RNAs of members of the alpha subdivision reveals a region of sharp change in the degree of homology in the 5′ half of the molecule. For example, the 5′ end of the wheat mitochondrial 5S RNA shows differences at 15 of 23 nucleotides when compared with the same region of the *Rb. sphaeroides* 5S RNA (65% dissimilarity); the next 28 positions show only 3 nucleotide differences (11% dissimilarity). The 3′ half of the molecule does not exhibit long stretches of nucleotide sequences with such extreme differences in homology, but several short segments near the 3′ end reveal close homologies between the wheat mitochondrial 5S RNA and the 5S RNAs of the purple photosynthetic bacteria (Fig. 1).

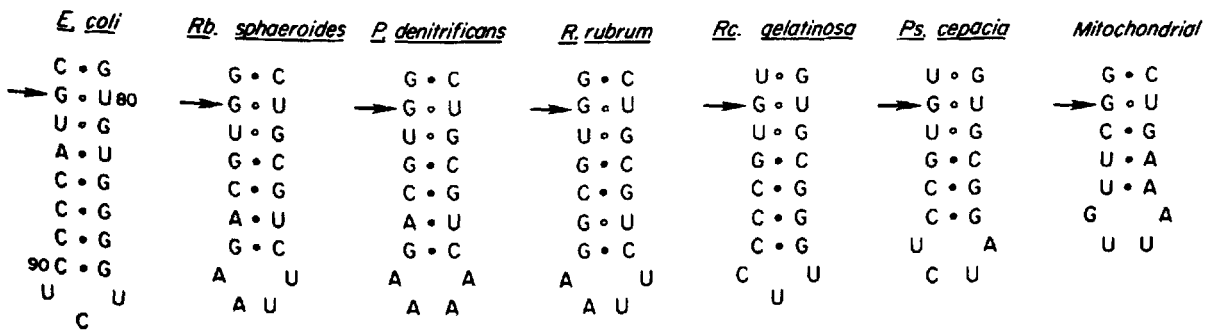

Fig. 3. Helix IV signatures. Arrow points to the universal GoU pair. *E. coli* 5S RNA shows the common helix length of procaryotic 5S RNAs

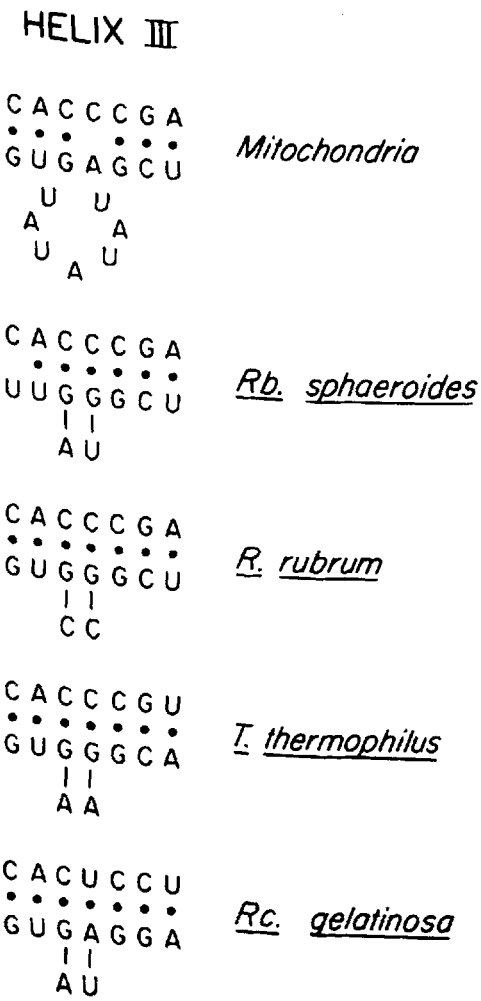

Fig. 4. Helix III homologies. The 5S RNAs of the alpha-subdivision species exhibit the closest base-pairing homologies to the plant mitochondria in this helix for the species shown. The RNAs of *Th. thermophilus* and the beta-subdivision species show increasingly divergent base-pairing schemes in this helix

Discussion

The evolutionary relationship between *Rb. sphaeroides*, *Rc. gelatinosa*, and *Ps. cepacia* is illustrated by the phylogenetic tree in Fig. 5. This tree was adapted from Stahl et al. (1984) and is based on 5S RNA sequence differences. The tree shows a large rift between *Rb. sphaeroides* and *Rc. gelatinosa*. The 5S RNAs from these organisms have only 65% sequence homology. This degree of homology is expected for members of unrelated genera. This phylogenetic rift determined by 5S RNA comparisons is analogous to what is observed by 16S ribosomal RNA cataloging (Gibson et al. 1979) and supports the rearrangement of the genus *Rhodopseudomonas* proposed by Imhoff et al. (1984). The tree also shows a close relatedness of *Rb. sphaeroides* to *P. denitrificans* (a 93% correlation) and of *Rc. gelatinosa* to *Ps. cepacia* (an 87% correlation). This again is in agreement with the 16S ribosomal RNA cataloging results.

Important secondary structural features useful as signatures are found in helices II, III, and IV. An anomalous 5S RNA structural feature observed previously in *P. denitrificans* is present also in the RNA of *Rb. sphaeroides*. This structure found in helix II (Fig. 2) consists of three mispaired bases at one end of the helix. An alternate pairing model is also presented in Fig. 2 that is justified by thermodynamic considerations but has no structural precedent in 5S RNA. Few 5S RNAs have mispaired bases in helix II. In those that do the most frequent example is an adenine–cytosine pair at the second position of the helix II extension. *Pseudomonas cepacia* 5S rRNA (Fig. 2) is typical in this regard. This is also seen in 5S rRNAs from *Streptococcus cremoris*, several mycoplasmas, and certain archaebacteria. The evolutionary acceptability of such an adenine–cytosine pair may be a special consequence of the conformation at the junction of the extension and the main portion of helix II.

A greater degree of overall sequence homology is found between the 5S RNA of the wheat mitochondrion and the 5S RNAs of the members of the alpha group than with the 5S RNAs of the members

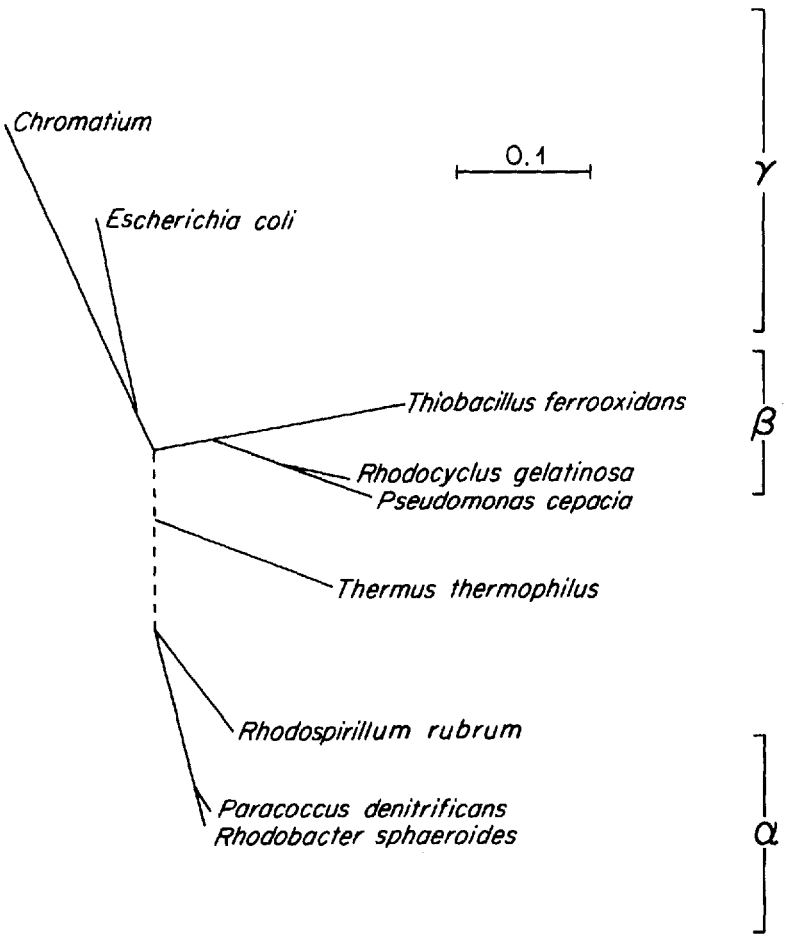

Fig. 5. Phylogenetic tree of the purple photosynthetic bacteria. Adapted from Stahl et al. (1984). The scale bar represents an evolutionary distance of 0.1 nucleotide changes per sequence position. Analyses that include 5S RNAs from distantly related eubacteria (not shown) suggest that the root of the tree lies within the dashed segments. It was not appropriate to include the mitochondrial 5S RNA in the construction of this tree because a significant number of its positions may not be homologous with those of other 5S RNAs.

of other subdivisions of the Gram-negative eubacteria (Table 1). This is true also for the mitochondrial 5S RNAs of maize (Chao et al. 1983) and soybean (Morgens et al. 1984), whose sequences are highly homologous to the wheat mitochondrial 5S RNAs. A strikingly high degree of sequence homology between the mitochondrial 5S RNA and the alpha-subdivision 5S RNAs is revealed in selected regions of the RNA (Table 2). This limited number of positions is sufficient in number to reconstruct the phylogenetic relations shown in Fig. 5. The alpha-subdivision regional homologies cluster closest to the mitochondrial 5S RNA, the beta and gamma homologies are farthest removed, and the *Th. thermophilus* 5S RNA regional homology is intermediate between the alpha and beta homologies. Similarities in several 5S RNA secondary structural signatures are also found. In helix IV, the alpha-subdivision species and *Rc. gelatinosa* 5S RNAs reveal one base-pair deletion and *Ps. cepacia* 5S RNA shows two base-pair deletions when compared with the generalized 5S RNA structure. Except in the 5S RNAs of the mycoplasmas and certain species of spirochetes and *Bacteroides* (J. Michalik and G.E. Fox, unpublished results), base-pair deletions in this helix are uncommon (Delihas et al. 1984) and therefore are appropriate group signatures. Similar signatures are found in mitochondrial 5S RNAs, which exhibit a three base-pair deletion in this helix (Spencer et al. 1981) (Fig. 3). Another signature shared by *Rb. sphaeroides, P. denitrificans, R. rubrum,* and *T. aestivum* mitochondria is the base pairing found in helix III (Fig. 4). The unusual six looped-out bases present in this helix of the wheat mitochondrial 5S RNA are not found in the 5S RNA of the soybean mitochondrial 5S RNA.

We conclude that the similarities in the structures of helices III and IV and the very high degree of homology exhibited by sequences proximal to these helices (Table 2) indicate a close evolutionary relationship between the plant mitochondria and the alpha subdivision of the purple photosynthetic bacteria. Interestingly, the first 25 nucleotides of the 5′ end of the wheat mitochondrial 5S RNA show only a 30%–35% homology with the 5′ ends of the alpha-subdivisional 5S RNAs, whereas the next 28 nu-

cleotides show approximately an 89% correlation. This extreme divergence in regional homology suggests that a gene rearrangement may have taken place within the 5S gene of the ancestral plant mitochondrion (or an ancestral progenitor bacterial species) in which the replacement of the first 25 nucleotides of the 5′ end of the 5S gene by another genetic segment resulted in a gene product with a partial but functionally acceptable base pairing in helix I and functional base pairing in helix II. It should be noted that helix I of the plant mitochondrial 5S RNAs has only six base pairs, whereas the 5S RNAs of most other sources have nine to ten base pairs in this helix (Delihas et al. 1984). Wolters and Erdmann (1984) show that the mitochondrial 5S RNAs can be approximately fitted to the alternate model of helix II shown in Fig. 2, a model specific to the RNAs of *P. denitrificans* and *Rb. sphaeroides*. Mitochondrial 5S gene rearrangements have been reported in soybean cells in culture (Morgens et al. 1984). A fusion with an unknown gene at the 3′ end of the mitochondrial 5S gene was detected. A comparison of the arrangement of ribosomal genes found in plant mitochondria shows that the order of genes differs from that found in eubacteria (Bonen and Gray 1980; Stern et al. 1982; Gray and Spencer 1983): The eubacterial ribosomal gene order is 16S–23S–5S, whereas the plant mitochondrial ribosomal gene order is 26S–18S–5S, with the 18S–5S genes far removed from the 26S gene.

If rearrangements occurred within the 5S gene during evolution of the plant mitochondrion, the regions of high homology depicted in Fig. 1 may represent some of the segments of the 5S RNA that are most crucial to the functions of the molecule and are likely not to survive major genetic rearrangements. These regions of high homology encompass mostly segments of the RNA that have high concentrations of the universally conserved positions (see Delihas et al. 1984), i.e., positions conserved in 5S RNAs of eubacteria, archaebacteria, eucaryotes, and organelles.

Uncertainties concerning the origins of the mitochondria have been discussed by Gray and Doolittle (1982). The conclusions presented in this paper strongly support the original proposal of John and Whatley (1975) that *P. denitrificans* and related organisms are likely bacterial candidates for mitochondrial origins. Questions concerning possible polyphyletic origins of the mitochondria (Gray et al. 1984) cannot be addressed with 5S rRNA data, because this molecule has been found only in the mitochondria of higher plants. Therefore it must be emphasized that the present results apply to plant mitochondria. Mitochondria from other sources may show evolutionary affinity with other bacterial groups (i.e., not with the alpha group).

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