The secondary structure and phylogenetic relationship deduced from complete nucleotide sequence of mitochondrial small subunit rRNA in yeast *Hansenula wingei*

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(Received 10 January 1996)

We have accomplished the nucleotide sequence of the 1537 bp mitochondrial gene coding for small subunit (SSU) rRNA of yeast *Hansenula wingei*, and also determined the 5'- and 3'-termini by S1 nuclease mapping. Eight universally conserved (U) elements of the SSU rRNA were identified. Comparison of U regions among five fungal mitochondrial SSU rRNAs shows the striking similarity between *H. wingei* and *Saccharomyces cerevisiae*. The construction of the secondary structure revealed a core structure similar to the counterpart of *Escherichia coli* 16S rRNA. The secondary structure also enabled us to specify seven variable (V) regions differing from those of other mitochondrial SSU rRNAs in size, sequence and possible secondary structure. Molecular phylogenetic evaluation based on U regions of five fungi indicates that mitochondria of *H. wingei* and *S. cerevisiae* diverged from the same lineage. This suggests that the evolution of mitochondria-encoded genes does not directly correlate with the alteration of mitochondrial genetic system: genome size, gene organization and codon usage.

INTRODUCTION

Large and small subunit (LSU and SSU) rRNA genes are universally present in mitochondrial genome. MtDNAs generally contain a copy of each LSU and SSU rRNA genes, whereas the sizes of rRNA genes vary widely among four eukaryotic kingdoms: animals, plants, protozoa and fungi. In fungi, for example, great variation in sizes of mitochondrial LSU and SSU rRNA genes has been observed among *Aspergillus nidulans* (Köchel and Küntzel, 1981; Netzker et al., 1982), *Podospora anserina* (Cummings et al., 1989a, b), *Saccharomyces cerevisiae* (Li et al., 1982; Sor and Fukuhara, 1983) and *Schizosaccharomyces pombe* (Lang et al., 1987; Trinkl et al., 1989). Despite of their size variations, mitochondrial LSU and SSU rRNA genes keep highly conserved regions which are identified by the similarity of primary and secondary structure (Gray et al., 1984; Cummings et al., 1989a). Thus, mitochondrial LSU and SSU rRNAs, such as those of archaeobacteria, eubacteria, plastids and eukaryotes, have been used for the construction of molecular phylogenetic trees.

Although the gene organization of mtDNA is essentially similar among fungi, there is still a significant difference; the mitochondrial genomes of all ascomycetous yeasts so far characterized completely lack NADH dehydrogenase (ND) subunit genes which are generally found in mtDNAs of filamentous ascomycetes. It had only been reported that the mitochondrial genome of yeast *Brettanomyces anomalus* could cross-hybridize with *A. nidulans* mitochondrial *ND1* gene (Hoeben and Clark-Walker, 1986). Contrary to general expectation, we have recently found that yeast *H. wingei* mtDNA distinctly encodes *ND4L* and *ND5* genes which are transcribed as a polycistronic mRNA (Okamoto et al., 1994). More recently, we have determined the complete sequence of mitochondrial genome (27694 bp) of *H. wingei* (Sekito et al., 1994, 1995). This shows that *H. wingei* mtDNA, like those of filamentous ascomycetes, contains a set of *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6* genes.

In this paper, we report the complete sequence and secondary structure of *H. wingei* mitochondrial SSU rRNA. In addition, we describe a phylogenetic tree based on comparison of the highly conserved regions of *H. wingei* mitochondrial SSU rRNA with those of two filamentous fungi, *A. nidulans* and *P. anserina*, and two yeasts, *S. cerevisiae* and *Sz. pombe*.

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MATERIALS AND METHODS

Preparation of mtRNA. Mitochondria were prepared as described by Okamoto et al. (1991). The isolated mitochondria were gently resuspended in 5 vol. of a lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.1 M NaCl, 1% SDS and 0.1 mg/ml Proteinase K). The lysate was incubated at 37°C for 30 min. After Phenol/Chloroform (1:1) extraction at 65°C for 15 min and subsequent ethanol precipitation, mtRNA pellet was dissolved in distilled water and stored at -85°C until use.

S1 nuclease mapping. For the 5'-end, the coding strand of *HinfI*-*NspI* fragment (from -130 to 49 in Fig. 2, 179 nucleotides) was end-labeled using [γ - 32 P]ATP and T4 polynucleotide kinase. The labeled probe was mixed with total mtRNA. The mixture was digested with S1 nuclease at 30°C for 2 h. The fragment protected from digestion was electrophoresed on a denaturing 6% polyacrylamide gel at 2000 V for 100 min simultaneously with reference ladders of M13mp18 ssDNA (Lanes G, A, T and C). For the 3'-end, the coding strand of *AlwNI*-*HinfI* fragment (from position 1490 to 1669 in Fig. 2, 180 nucleotides) was end-labeled using [α - 32 P]ddATP and terminal transferase. The following steps were the same as in the case of the 5'-terminus.

Computer analysis. DNA sequence analysis was carried out by using CLUSTAL programs (Higgins and Sharp, 1988).

RESULTS AND DISCUSSION

Primary structure of *H. wingei* mitochondrial SSU rRNA. As shown in Fig. 1, the gene coding for SSU

rRNA in *H. wingei* mtDNA is located between Pro-tRNA gene and Val-tRNA gene. At first, the boundaries of coding region were estimated by the sequence similarity to the corresponding gene of *S. cerevisiae* (Li et al., 1982). The nucleotide sequence of *H. wingei* mitochondrial SSU rRNA gene and its flanking regions is shown in Fig. 2. A nonanucleotide sequence, ATATAAGAA (position -62 to -54), significantly similar to mitochondrial promoter motif of *S. cerevisiae* (Osinga and Tabak, 1982; Christiansen et al., 1982), appears to be an initiation signal of transcription for the SSU rRNA gene.

To identify the boundary of the SSU rRNA gene, we directly determined the 5'- and 3'-termini of the mature transcript by S1 nuclease mapping (Fig. 3). From the signal intensity of bands indicating fragments protected from S1 nuclease digestion, we assigned the 5'-end at position 1 and the 3'-end at position 1537 as shown in Fig. 2, a total length of 1537 bp. The 5'- and 3'- regions are flanked by AT-rich sequences. Val-tRNA is encoded 72 bp downstream of the SSU rRNA gene. In *S. cerevisiae* and *Sz. pombe* mitochondria, tRNA sequence acts as a primary signal for RNA processing (Wolf and Del Giudice, 1988). Although there is no direct evidence, the Val-tRNA also may play a similar role in RNA processing in *H. wingei*.

Universally conserved regions in SSU rRNA. SSU rRNA contains a universally conserved secondary structure core that has an important function in protein synthesis (Stiegler et al., 1981). The minimal core is composed of eight non-contiguous elements of primary sequence. The U elements permit a precise alignment for comparative analysis of primary structure, and for construction of phylogenetic tree. As shown in Table 1, the U regions of *H. wingei* mitochondrial SSU rRNA, U1-U8, were compared with other counterparts (Gray et al., 1984;

Fig. 1. Structure of *H. wingei* mitochondrial SSU rRNA gene and strategy for S1 nuclease mapping. Position 1 corresponds to the first nucleotide of the mature SSU rRNA transcript. The SSU rRNA gene is located between Pro-tRNA gene (5'-upstream) and Val-tRNA gene (3'-downstream). Black parts in a white box representing the SSU rRNA gene denote U regions. Dotted boxes show two restriction fragments, *HinfI*-*NspI* and *AlwNI*-*HinfI*, used as probes for determination of 5'- and 3'-termini by S1 nuclease mapping. Restriction cleavage sites are abbreviated as follows: A, *AlwNI*; Ba, *BamHI*; Bc, *BclI*; Hf, *HinfI*; Hd, *HindIII*; M, *MunI*; Ni, *NsiI*; Np, *NspI*; P, *PstI*.

Fig. 2. Nucleotide sequence of SSU rRNA gene and its flanking regions in *H. wingei* mtDNA. The non-coding (transcript-like) strand is shown. Numbering starts at a base corresponding to the first nucleotide of mature SSU rRNA. Arrowheads indicate the 5'- and 3'-termini of SSU rRNA gene identified by S1 nuclease mapping. The U1-U8 sequences in the SSU rRNA are underlined. A possible transcription initiation site from position -62 to -54, similar to a consensus motif for mtDNA promoters in yeasts, is marked with asterisks. This sequence has been deposited in the DDBJ database under accession No. D49702. The sequences coding for Pro- and Val-tRNA have been published elsewhere (Okamoto et al., 1992).

Fig. 3. Mapping of the 5'- and 3'-termini of *H. wingei* mitochondrial SSU rRNA gene. Arrows indicate bands displaying the strongest signal. Note that the bands are equally 48 nucleotides long.

Table 1. Sequence homology (%) between the U regions^a of *H. wingei* mitochondrial SSU rRNA and other counterparts

U (positions) ^b	ANI	PAN	SCE	SPO
U1 (5-64)	66.7	68.3	76.7	75.4
U2 (310-362)	79.2	78.0	88.7	82.4
U3 (520-604)	71.6	68.8	81.0	62.9
U4 (719-795)	69.9	73.3	80.0	65.8
U5 (831-948)	72.9	69.8	78.0	71.7
U6 (1108-1173)	73.8	70.8	83.8	72.7
U7 (1253-1392)	62.7	67.5	78.6	66.1
U8 (1457-1514)	68.6	76.6	86.8	70.4

^aThe U regions of *H. wingei* mitochondrial SSU rRNA were selected with reference to the conserved secondary structure core identified by Stiegler et al. (1981).

^bThe positions of the U regions correspond to the nucleotide numbers in Fig. 2.

Abbreviations: ANI, *A. nidulans*; PAN, *P. anserina*; SCE, *S. cerevisiae*; SPO, *Sz. pombe*.

Cummings et al., 1989a). The combined U1-U8 sequence corresponds to a total of 668 nucleotides (containing gap sequences) in the SSU rRNA. The U2 sequence, forming

a part of 5'-domain of the SSU rRNA, has the highest similarity to all other equivalents. The average of the nucleotide sequence identities in eight U regions indicates that the mitochondrial SSU rRNA of *H. wingei* is more closely related to that of *S. cerevisiae* than to those of *A. nidulans*, *P. anserina* and *Sz. pombe*.

Secondary structure of SSU rRNA. *E. coli* 16S rRNA was used as a standard model for comparison with mitochondrial SSU rRNAs. The comparison postulates that these rRNA molecules with different primary structure and same functional properties have essentially similar secondary structure. Mitochondrial SSU rRNAs, though

have extreme variability in size ranging about from 600 nucleotides in *Chlamydomonas* (Boer and Gray, 1988) to 2000 nucleotides in wheat (Spencer et al., 1984), can be essentially described by the prokaryotic *E. coli* structural model. The secondary structure models also specify U and V regions. The U region has an important role in protein synthesis, so that it shows highest homology in primary sequence. On the other hand, V region remarkably varies in size, sequence and structure against the *E. coli* standard structure. Thus, the secondary structure diagram of rRNA can offer the cardinal information for its structural characteristics and phylogenetic relationship.

As shown in Fig. 4A, the secondary structure of *H.*

Fig. 4. Diagrams of secondary structure for *H. wingei* mitochondrial SSU rRNA (A) and *E. coli* 16S rRNA (B). The secondary structure of *H. wingei* was constructed from the DNA sequence by comparison with that of *E. coli* (Gutell, 1993). A: U, G:C and G:U base pairings are represented by bars; G:A pairing is denoted with a dot. U1-U8 and V1-V7 in the *H. wingei* model indicate universal and variable regions, respectively.

wingei mitochondrial SSU rRNA was constructed by direct comparison with that of *E. coli* 16S rRNA (Gutell, 1993). The secondary folding primarily depends on the ability of base pairing by hydrogen bond. Standard base pairings, A:U and G:C (represented by bars), are the main force for folding the secondary structure. G:U base pairing is possible (also depicted by bars). Other non-canonical interaction, G:A pairing is distorted in the double helix formation (denoted with dots). The presence of many non-canonical G:A and G:U is a general characteristic of many SSU rRNAs (Ehresmann et al., 1990). As it is evident from Fig. 4B, the general structure of *E. coli* 16S rRNA has very close resemblance with that of *H. wingei* mitochondrial SSU rRNA.

The core structure of *H. wingei* mitochondrial SSU rRNA is comprised of eight non-contiguous segments (U1-U8) of primary sequence, and held together by hydrogen bondings like the universal core structure in *E. coli* 16S rRNA. The alignment of homologous segments of the core is relatively easy, since each portion is exactly defined by secondary structure position. V regions, V1-V7, in *H. wingei* mitochondrial SSU rRNA differ markedly in size, primary sequence and possible secondary structure. V3 (position 605 to 616 in Fig. 2) is very similar to SV7 of *P. anserina* mitochondrial SSU rRNA (Cummings et al., 1989a). The region is rather shorter than the counterpart in *E. coli* 16S rRNA. V4 (position 954 to 975 in Fig. 2) is a variant region corresponding to that of *E. coli* (indicated by an arrow in Fig. 4B). SV3, *P. anserina* counterpart for V4 of *H. wingei*, is unstructured region (Cummings et al., 1989a). V5 and V6 are additional regions which are absent from other SSU rRNAs, suggesting the specific characteristics of *H. wingei* or a closely related lineage of *Hansenula* species.

Phylogenetic analysis. Based on the primary sequence of U regions, *H. wingei* mitochondrial SSU rRNA has a striking resemblance to that of *S. cerevisiae*. To know the lineage of fungal mitochondria, we aligned a set of U1-U8 sequences from *E. coli*, *H. wingei*, *A. nidulans*, *P. anserina*, *S. cerevisiae* and *Sz. pombe*. *E. coli* is an outgroup to root to phylogenetic tree. Each alignment is comprised of a total 668 nucleotides with gap sequences. These sequences were analyzed with CLUSTAL, multiple alignment programs (Higgins and Sharp, 1988), calculating the number of nucleotide substitution by 2-parameter method (Kimura, 1980), constructing the phylogenetic tree by neighbor-joining method (Saitou and Nei, 1987) and estimating the degree of error in the tree by bootstrap analysis (Felsenstein, 1985). The upper right triangle in Table 2 shows distance data derived from the U1-U8 alignment without gap sequence. A similar data was obtained using the alignment with gap sequence (lower left triangle in Table 2). Based on the former data, we constructed a network relationship of five fungal mitochondria and *E.*

Table 2. Distance matrix for SSU rRNAs

	ECO	HWI	ANI	PAN	SCE	SPO
ECO		0.3855	0.3161	0.3290	0.3500	0.3419
HWI	0.3898		0.3081	0.3145	0.1806	0.3274
ANI	0.3241	0.3152		0.1129	0.2871	0.2968
PAN	0.3338	0.3161	0.1173		0.3016	0.3113
SCE	0.3583	0.1883	0.3002	0.3072		0.3258
SPO	0.3480	0.3291	0.3027	0.3102	0.3302	

Upper right triangle: distance data based on the alignment of U1-U8 regions without gap sequences. Lower left triangle: data based on the alignment of U1-U8 regions with gap sequences.

Abbreviations are the same as in Table 1 except for ECO, *E. coli* and HWI, *H. wingei*.

Fig. 5. Phylogenetic relationship deduced from evolutionary distances in the U sequences among SSU rRNAs. The *E. coli* 16S rRNA sequence was used as an outgroup. Branch lengths of the tree are proportional to the inferred mutational distance (see scale). Numerals on branches refer to the percentage of times a particular branch was found in 1000 bootstrap samples. Abbreviations are the same as in Table 1 except for ECO, *E. coli* and HWI, *H. wingei*.

coli as shown in Fig. 5. The grouping of budding yeasts are significantly separated from that of filamentous fungi (at a bootstrap level of 100%). However, a short branch that includes the above two groupings is not verified (at 55% level). Like the estimation from the similarity of the primary sequences in U regions (Table 1), the tree at least clearly indicates that both *H. wingei* and *S. cerevisiae* mitochondria diverged from the common root. The branch-

ing order among five fungal mitochondrial representatives is essentially consistent with the phylogenetic trees deduced from mitochondrial LSU rRNAs (Lang et al., 1987) and from restriction fragment length polymorphism of yeast mtDNAs (Okamoto et al., 1991). Although two mitochondria are grouped into a related lineage which are relatively remote from other fungi, mitochondrial genome of *H. wingei* differs markedly from that of *S. cerevisiae* in size, structure, gene organization and genetic code. This suggests that the alteration of mitochondrial genetic system does not directly correlate with the evolution of mitochondria-encoded genes.

This work was partially supported by grants from the Ministry of Science, Culture, Sports and Education, Japan, and by a grant from Electric Foundation of Chugoku. K. O. received a fellowship from the Japan Society for Promotion of Science for Japanese Junior Scientists.

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