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Location and Structure of the *var1* Gene on Yeast Mitochondrial DNA: Nucleotide Sequence of the *40.0* Allele

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Summary

Alleles of the *var1* locus on yeast mitochondrial DNA specify the size of var1 ribosomal protein. We report the nucleotide sequence of a *var1* allele that determines the smallest var1 protein. It contains an open reading frame of 396 codons, which we identify as the structural gene for var1 protein. The var1 protein specified by this allele has an amino acid composition in close agreement with that predicted by the DNA sequence. The *var1* coding region is highly unusual: it is 89.6% AT and contains a 46 bp GC-rich palindromic cluster that accounts for 38% of the total GC residues. Our results strongly suggest that like mammalian mitochondria but unlike those from Neurospora, yeast mitochondria use AUA as a methionine codon. Comparison with the sequence of a *var1* allele specifying a larger protein suggests that some size polymorphism of var1 protein results from in-frame insertions of a variable number of AAT (Asn) codons.

Introduction

The mitochondrial genome of Saccharomyces cerevisiae has been well studied, since it lends itself to both genetic and physical analysis. At the nucleotide sequence level, the genes of six proteins have been analyzed thus far—cytochrome oxidase subunits I to III (Coruzzi and Tzagoloff, 1979; Fox, 1979; Bonitz et al., 1980b; Thalenfeld and Tzagoloff, 1980), cytochrome b (Nobrega and Tzagoloff, 1980) and ATPase subunits 9 and 6 (Macino and Tzagoloff, 1979, 1980)—as well as the 15S ribosomal RNA gene (Sor and Fukuhara, 1980) and numerous transfer RNA genes. In addition to the above genes, eight open but unassigned reading frames have been identified (Bonitz et al., 1980b; Dujon, 1980; Lazowska et al., 1980, 1981; Nobrega and Tzagoloff, 1980; Coruzzi et al., 1981), and all but one occur in introns of other genes.

Var1 is a mitochondrial translation product that is associated with the small subunit of yeast mitochondrial ribosomes (Groot et al., 1979; Terpstra and Butow, 1979; Terpstra et al., 1979). Var1 protein has at least two unusual properties: it is polymorphic in different yeast strains (Douglas and Butow, 1976; Strausberg and Butow, 1981); and crosses between strains with two different var1 forms generate nonparental species, and do so with the characteristics of an asymmetric recombination event whereby "short" forms of the protein are converted preferentially to "long" forms (Strausberg et al., 1978; Strausberg and Butow, 1981).

The size polymorphism of var1 proteins and their recombination behavior in crosses have allowed us to define a series of *var1* alleles (alleles are designated as *var1*[*n*] or [*n*], where n is the apparent size, in kilodaltons (kd), of the var1 protein present in that allele; the proteins are designated as var1[n kd]), which we have mapped to a 2.6 kb segment of the yeast mitochondrial genome between *ery* (21S rRNA) and *oli1* (ATPase 9). This region was previously termed the *var1* determinant (Perlman et al., 1977; Lopez et al., 1981; Strausberg and Butow, 1981). It contains strain-specific insertions and deletions, first detected by restriction mapping, some of which correlate with different forms of the var1 protein (Strausberg et al., 1978; Vincent et al., 1980; Vincent, 1982).

Tzagoloff et al. (1980) have reported the nucleotide sequence of the region between *ery* and *oli1* in a petite strain (DS401, derived from wild-type strain D273-10B) that produces a var1 protein of intermediate size. They concluded that the structural gene for var1 protein could not be present in this region in a simple form because the region has a much higher AT content than any bona fide mitochondrial gene sequenced, and because no open reading frame exists that starts with a methionine codon and that extends for more than 37 amino acids. We recently attempted to rationalize their findings with our genetic and physical mapping data (Butow et al., 1982).

We now report the nucleotide sequence of the *var1*[*40.0*] allele, which contains the smallest var1 protein seen to date, and compare this sequence with that of the [*42.0*] allele in DS401 (Tzagoloff et al., 1980), which specifies a var1 protein about 2 kd larger. Our sequence in the *var1* determinant region contains an open reading frame that starts with a methionine codon and runs sequentially for 396 amino acids to an ochre terminator. The amino acid composition predicted by the DNA sequence agrees closely with that determined from analysis of var1 protein, which we have purified to apparent homogeneity. Thus we identify this sequence as the structural gene for var1 protein. Its base composition of nearly 90% AT, with about one third of the GC content present as a single 46 bp cluster, makes it the most AT-rich gene we know of in nature. Results to be presented elsewhere (M. E. S. Hudspeth, R. D. Vincent, L. O. Treisman, D. S. Shumard, P. S. Perlman and L. I. Grossman, manuscript in preparation) describe DNA inser-

tions within the reading frame of *var1[40.0]* in strains producing larger var1 proteins.

Results

Restriction Map of Petite A17-10 and Sequencing Strategy

The mitochondrial DNA (mtDNA) of petite A17-10 was shown by Vincent et al. (1980) to be a 5.3 kb head-to-tail repeat. From transmission analysis and zygotic gene-rescue analysis (Strausberg and Butow, 1977; Vincent et al., 1980; Lopez et al., 1981), we know that A17-10 mtDNA contains the entire *var1* determinant region located within a 2.7 kb fragment corresponding to Hinc II band 10 of the wild-type genome. The restriction map of A17-10 mtDNA has been extended with ten additional enzymes to show fine structure (Hudspeth et al., manuscript in preparation), and a summary of that map for Hinc II band 10 is shown in Figure 1.

The strategy used for sequencing the open reading frame is shown in Figure 2. This figure shows the fragments that were labeled at either their 5′ or 3′ termini and the manner in which the labeled ends were made asymmetric. All of the sites labeled for sequencing within the coding region were crossed at least once from other labeled ends, except for the Bcl I site.

Nucleotide Sequence of the *var1[40.0]* Allele

The sequence of the open reading frame of the *[40.0]* allele is shown in Figure 3. It encodes 396 amino acids, or a protein of molecular weight 46,786. This value is in reasonable agreement with a molecular weight of 40,000 estimated by SDS-polyacrylamide gel electrophoresis for the protein specified by the *var1[40.0]* allele (Perlman et al., 1977; Strausberg and Butow, 1981). We note that underestimates of protein size by SDS-polyacrylamide gel electrophoresis for other mitochondrially encoded proteins have been observed. For instance, sizes of human cytochrome oxidase subunits I–III were underestimated by 21%–40% compared with the protein sizes derived from DNA sequencing (Hare et al., 1980; Anderson et al., 1981). Similar underestimates have been seen for yeast mitochondrial proteins (Groot et al., 1978; Bonitz et al., 1980b).

The reading frame in *var1* is on the same strand as most other genes on yeast mtDNA. Analysis of *var1* transcripts (H. P. Zassenhaus, Y. Hannon and R. A. Butow, manuscript in preparation) confirms this assignment and shows that transcription is in the direction *oli1* to *ery*. However, unlike other yeast mitochondrial genes and unassigned open reading frames, the *var1* sequence is 89.6% AT, with more than one third of the GC concentrated in a 46 bp palindromic cluster starting at nucleotide 198 in Figure 3.

Purification of the var1[40.0 kd] Protein, and Amino Acid Analysis

For amino acid analysis, we purified var1 protein in essentially homogeneous form directly from SDS-polyacrylamide gels (see Experimental Procedures). The var1 protein is tightly associated with the 38S mitochondrial ribosomal subunit (Terpstra et al., 1979), and therefore can easily be purified away from the bulk of mitochondrial proteins. Figure 4 shows that var1 protein is well resolved from the other 38S ribosomal proteins by both one- and two-dimensional gel electrophoresis. Figure 4 also shows a typical preparation of var1 protein, purified from 38S subunits, that migrates as a single band on an 11% SDS-polyacrylamide gel. Displayed on the same gel is a profile of mitochondrial translation products labeled in vivo in the presence of cycloheximide with $^{35}SO_4^{2-}$ (Douglas and Butow, 1976), showing that the labeled var1 comigrates with purified var1 protein and with var1 from the 38S ribosomal subunit. The determination of amino acid composition of purified var1 protein was carried out as described in the Experimental Procedures; the results are presented in Table 1.

Assignment of the Open Reading Frame as var1

The amino acid sequence specified by the open reading frame is also shown in Figure 3; its composition is summarized in Table 1. For translation we have used the previously noted modifications of the universal

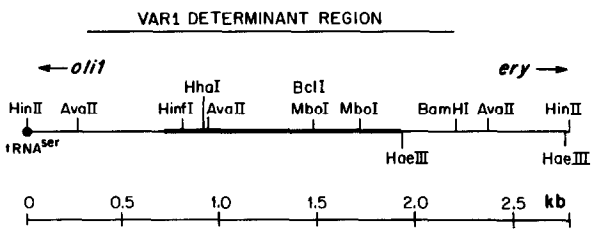

Figure 1. Restriction Map of the *var1* Determinant Region

Hinc II fragment 10 of wild-type mtDNA is shown with selected restriction sites. $tRNA^{Ser}$ is from Tzagoloff et al. (1980). The *var1* determinant region was defined by gene rescue with petite mutants; the ends of the region between Ava II and Hinf I, and between Hae III and Bam HI, are not defined. Thick line: open reading frame.

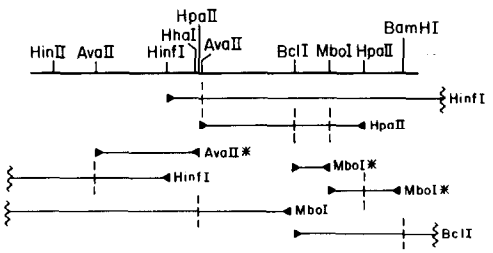

Figure 2. Labeling Strategy for Sequencing *var1[40.0]*

A portion of Hinc II fragment 10 (Figure 1) is shown. The fragments were digested with the indicated enzymes and labeled at their 5′ or 3′ ends, and the labeled ends were segregated by strand separation (asterisks) or by secondary cleavages (vertical dashed lines). Vertical wavy lines: the fragment extends beyond the map portion shown.

```
   *       met lys leu lys leu leu asn met ile leu ser met met asn lys thr asn asn asn asn asn ile
  TAAAAAATAATG AAA TTA AAA TTA TTA AAT ATA ATT TTA TCA ATA ATA AAT AAA CTT AAT AAT AAT AAT AAT ATT   75

ile ile asn asn thr leu asp ser leu met asn lys lys leu leu leu lys asn met leu leu asp met asn asn
ATT ATT AAT AAT CTA TTA GAT TCA TTA ATA AAT AAG AAA TTA TTA TTA AAG AAT ATA TTA TTA GAT ATA AAT AAT  150

lys lys met asn asn met lys arg met leu asn asn asn asn met asn pro ala gly ala asn pro val val his
AAA AAA ATA AAT AAT ATA AAA AGA ATA TTA AAT AAT AAT AAT ATA AAC CCC GCG GGC GCC AAT CCG GTT GTT CAC  225

arg ile gly pro ala gly asn ile asn asn lys leu gln his leu asn asn met asn asn trp asn thr gln ile
CGG ATT GGT CCC GCG GGG AAT ATT AAT AAT AAA TTA CAA CAT TTA AAT AAT ATA AAT AAT TGA AAT CTA CAA ATT  300

tyr asn tyr asn lys asn met glu ile met asn thr met asn asp lys leu ile asn lys leu leu tyr lys met
TAT AAT TAT AAT AAA AAT ATA GAA ATT ATA AAT ACT ATA AAT GAT AAA TTA ATT AAT AAA TTA TTA TAT AAA ATA  375

met thr leu lys leu asn asn met asn ile asn lys ile ile met ser lys thr ile asn gln his ser leu asn
ATA ACT TTA AAA TTA AAT AAT ATA AAT ATT AAT AAA ATT ATT ATA AGT AAA CTT ATT AAT CAA CAT AGT TTA AAT  450

lys leu asn ile lys phe tyr tyr tyr asn asn asp ile asn asn asn asn asn asn asn asn tyr tyr met asn
AAA TTA AAT ATT AAA TTT TAT TAT TAT AAT AAT GAT ATT AAT AAT AAT AAT AAT AAT AAT AAT TAT TAT ATA AAT  525

met met asn lys leu met asn ile met asn asn asn met asn asn asn leu cys asn ile leu ser tyr tyr tyr
ATA ATA AAT AAA TTA ATA AAT ATT ATA AAT AAT AAT ATA AAT AAT AAT TTA TGT AAT ATT TTA AGT TAT TAT TAT  600

asn lys lys val thr ile glu pro ile lys leu ser tyr ile tyr leu asn ser asp ile phe ser lys tyr ile
AAT AAA AAA GTA ACT ATT GAA CCT ATT AAA TTA TCA TAT ATT TAT TTA AAT AGT GAT ATT TTT AGT AAA TAT ATT  675

ser leu asn asp met asp lys tyr asn asn gly ile leu thr asn tyr gln arg met leu asn asn ile met pro
AGT TTA AAT GAT ATA GAT AAA TAT AAT AAT GGT ATC TTA ACT AAT TAT CAA CGT ATA TTA AAT AAT ATT ATG CCT  750

lys leu asn asp his asn ile ser met asn tyr ile asn asn ile asn asn ile asn asn asn lys tyr asn asn
AAA TTA AAT GAT CAT AAT ATT TCT ATA AAT TAT ATT AAT AAT ATT AAT AAT ATT AAT AAT AAT AAA TAT AAT AAT  825

met ile asn leu leu asn asn asn asn asn ile asn asn asn asn asn tyr asn asn asn asn asn asn tyr ile
ATA ATT AAT TTA TTA AAT AAT AAT AAT AAT ATT AAT AAT AAT AAT AAT TAT AAT AAT AAT AAT AAT AAT TAT ATT  900

gly asn ile asn asn ile tyr asn asn met thr ile asp asn ile pro met asp ile leu met tyr lys tyr leu
GGT AAT ATT AAT AAT ATT TAT AAT AAT ATA ACT ATT GAT AAT ATT CCT ATA GAT ATT TTA ATA TAT AAA TAT TTA  975

val gly trp ser ile lys phe lys gly arg leu ser asn asn asn gly arg thr ser thr thr asn leu leu asn
GTT GGT TGA TCT ATT AAA TTT AAA GGT AGA TTA AGT AAT AAT AAT GGT AGA ACT AGT ACA CTT AAT TTA TTA AAT 1050

gly thr phe asn asn lys lys tyr leu trp ser asn ile asn asn asn tyr lys leu asn tyr ile pro ser asn
GGT ACT TTT AAT AAT AAA AAA TAT TTA TGA AGT AAT ATT AAT AAT AAT TAT AAA TTA AAT TAT ATC CCT TCT AAT 1125

his asn leu tyr asn asn ser asn ile asn lys asn gly lys tyr asn ile lys val lys leu asn phe ile  *
CAT AAT TTA TAT AAT AAT TCT AAT ATT AAT AAA AAT GGT AAA TAT AAT ATT AAA GTT AAA TTA AAC TTT ATT TAA 1200
```

Figure 3. Nucleotide Sequence of the *var1[40.0]* Open Reading Frame

The sequence shown is of the nontranscribed strand, with the 5′ end at the upper left. Asterisks: ochre triplets. The sequence is translated with the yeast mitochondrial genetic code (Bonitz et al., 1980a). In addition, ATA has been translated as methionine (see text). Underlining: substitutions compared with the *[42.0]* allele sequence. Double underlining: insertions. Brackets: acceptor regions for the allele-specific insertions (see text).

genetic code (Bonitz et al., 1980a) that UGA signifies tryptophan and CUN signifies threonine, as well as the assumption (see below) that AUA encodes methionine. As anticipated from its base composition, the open reading frame specifies a low proportion of amino acids encoded by GC-rich codons and is well represented by amino acids encoded by AT-rich codons. Particularly striking is the extraordinary number of asparagines, which represent nearly one third of the total amino acid residues.

A comparison of the amino acid composition of purified var1[40.0 kd] protein with the composition specified by the DNA sequence shows a close correspondence between the predicted and experimentally determined values (Table 1). Using a simple algorithm (Cornish-Bowden, 1978) to estimate the extent of amino acid sequence identity between two proteins of equal length from their amino acid composition, we calculate a 93% probability of sequence identity between the protein specified by the *var1* open reading frame and the var1[40.0 kd] protein. This agreement, considered particularly in light of the very unusual amino acid composition predicted by the *var1* sequence, argues strongly that the var1 protein is encoded by the open reading frame in the *var1* determinant region.

Additional support for the assignment to the open reading frame comes from preliminary radiochemical sequencing of the var1[42.0 kd] protein labeled in vivo with $^{35}SO_4^{2-}$ (H. P. Chiang and R. A. Butow, unpublished results), showing a correspondence between the position of methionine residues at the amino end (assuming AUA as a methionine codon; see below) and the distribution of radioactive sulfur.

Assignment of AUA As a Methionine Codon

Translation of the open reading frame with the standard genetic code that has been modified solely by the assignment of UGA as tryptophan and of CUN as threonine (Bonitz et al., 1980a) provides only two significant disagreements (>5 mean deviations) with the measured amino acid composition: methionine is

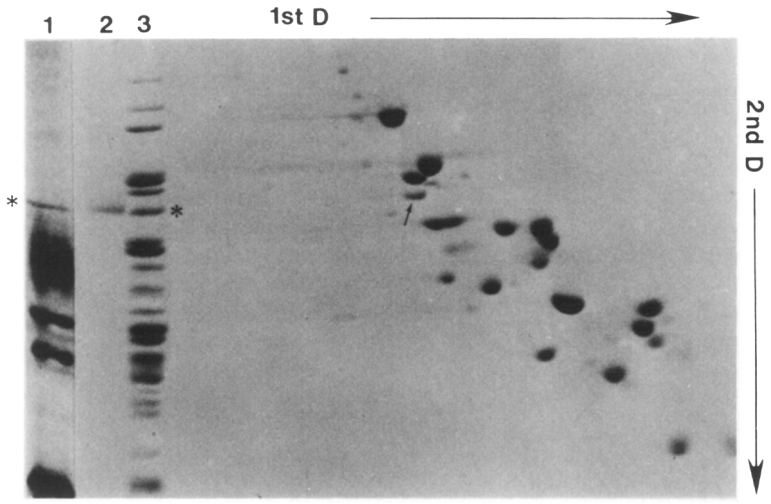

Figure 4. Electrophoretic Analysis of Purified var1 Protein: Comparisons with 38S Mitochondrial Ribosomal Proteins and in Vivo–Labeled Mitochondrial Translation Products

(Lanes 1–3) Samples were subjected to electrophoresis on an 11% SDS gel and autoradiographed. (Lane 1) Mitochondrial translation products labeled in vivo with $^{35}SO_4^{2-}$; (lane 2) purified var1 protein; (lane 3) 38S mitochondrial ribosomal proteins. Except for lane 1, proteins were visualized by staining with Coomassie blue. Two-dimensional gel electrophoresis of the 38S mitochondrial ribosomal proteins was carried out as described in the Experimental Procedures. Lanes 1–3 were run on the same gel as the second dimension (2nd D) of the two-dimensional gel. Asterisks and arrow: var1.

Table 1. Amino Acid Composition of Purified var1 Protein Agrees Closely with the Composition Predicted from the *var1* DNA Sequence

	Composition (mole %)	
Amino Acid	Calculated from DNA	Determined from Protein
Alanine	0.8	1.3
Arginine	1.3	1.1
Aspartic acid + asparagine	35.3	36.0
Glycine	2.6	2.7
Glutamic acid + glutamine	1.6	2.3
Histidine	1.3	1.3
Isoleucine	10.9	10.5
Leucine	10.6	11.3
Lysine	9.6	9.4
Methionine	8.6	6.9
Phenylalanine	1.3	1.4
Serine	4.2	3.9
Threonine	3.4	3.2
Tyrosine	7.3	7.4
Valine	1.3	1.4

The amino acid composition derived from DNA was translated with the assumptions that CUN encodes threonine and AUA encodes methionine (see text). Cysteine, proline and tryptophan were not determined in the protein sequence and were not used in the calculation of composition (mole %) from DNA. The values determined from protein are averaged from 15 and 24 hr hydrolyses.

predicted to constitute 0.5 mole % and is found to be 6.9 mole %, and isoleucine is predicted to be 18.4 mole % and is found to be 10.5 mole %. Both discrepancies are resolved if AUA is assigned as a methionine rather than an isoleucine codon (Table 1).

The assignment of AUA to methionine, which increases the symmetry of the AUN box of the genetic code table, was previously suggested for the human and bovine mitochondrial genetic codes by comparison of the amino acid sequences from DNA with the amino acid sequence of bovine cytochrome oxidase subunit II (Barrell et al., 1979; Young and Anderson, 1980). We thus suggest that AUA represents methionine in yeast mitochondria as well. As shown in Table 2, AUA is used for 31 of the 33 residues assigned as methionine, while AUG is used only once internally. The use of AUA for Met by other yeast mitochondrial genes, but not by Neurospora mtDNA (Browning and RajBhandary, 1982), is examined in the Discussion.

Other Codon Usage

Table 2 also lists the use of other codons in *var1*, and compares *var1* with other yeast mitochondrial genes, and with mitochondrial open reading frames unassigned to any proteins. In all of the data, the clear pattern is for the use of the most AU-rich codons in both genes and open reading frames, consistent with the low GC content of yeast mtDNA. Within this overall pattern, several additional trends can be seen. It has already been noted that arginine codons of the CGN series are found in open reading frames, but have not been seen in established genes, and that phenylalanine codons are about equally divided between UUU and UUC in genes, but strongly biased toward UUU in open reading frames (Bonitz et al., 1980b). A similar bias is seen here in the use of AUG and AUA codons for methionine. The use of codons by *var1* resembles that of open reading frames by these criteria. In addition, we note that where a choice is possible, the *var1* gene uses codons ending in U rather than A, although the nontranscribed strand contains a 10% excess of A over T.

Comparison with the [*42.0*] Allele Sequence

Our sequence of *var1*[*40.0*] differs in three ways from the sequence reported for the [*42.0*] allele by Tzagoloff et al. (1980).

Table 2. Codon Usage in the *var1* Open Reading Frame

Amino Acid	Codon	Genes[a]	ORF[b]	*var1*	Amino Acid	Codon	Genes[a]	ORF[b]	*var1*
Ala	GCA	53	30	0	Lys	AAA	31	352	35
	U	66	58	0		G	1	26	2
	C	5	10	1					
	G	2	7	2	Met[c]	AUA	7	133	31
						G	65	49	2
Arg	AGA	37	121	3					
	G	0	7	0	Phe	UUU	71	136	5
	CGA	0	0	0		C	60	19	0
	U	0	14	1					
	C	0	1	0	Pro	CCA	35	26	0
	G	0	1	1		U	39	63	4
						C	2	5	2
Asn	AAU	62	356	124		G	0	8	1
	C	9	26	2					
					Ser	UCA	71	71	3
Asp	GAU	41	137	10		U	34	68	4
	C	2	9	0		C	1	10	0
						G	0	3	0
Cys	UGU	12	53	1		AGU	15	67	9
	C	1	1	0		C	0	3	0
Gln	CAA	29	69	4	Thr	ACA	51	76	1
	G	4	7	0		U	34	62	7
						C	1	12	0
Glu	GAA	37	105	2		G	0	17	0
	G	3	16	0		CUA	14	24	2
						U	2	21	3
Gly	GGA	28	52	0		C	0	1	0
	U	88	120	8		G	2	0	0
	C	0	6	1					
	G	6	14	1	Trp	UGA	36	47	3
						G	0	6	0
His	CAU	47	65	4					
	C	3	3	1	Tyr	UAU	73	209	28
						C	13	17	0
Ile[c]	AUU	149	270	40					
	C	26	22	2	Val	GUA	76	89	1
						U	42	66	4
Leu	UUA	224	338	41		C	4	4	0
	G	2	13	0		G	6	10	0

Data are given as number of occurrences of the codons in the genes listed.

[a] Data for genes are for *oli1*, *oli2*, *oxi1*, *oxi2* and the exons of *oxi3* and *cob-box*.

[b] Data for the open reading frames (ORF) are from seven intron-encoded reading frames in omega, *oxi3* and *cob-box*, and one free-standing open reading frame near *oxi1* (Coruzzi et al., 1981).

[c] AUA is assigned as a methionine codon (see text).

First, in both the *var1*[*40.0*] and [*42.0*] alleles, the orientation of the small Mbo I–Mbo I fragment (Figure 1) is that given by the sequence of Figure 3, and not the inverted orientation given by Tzagoloff et al. (1980). This conclusion is based on our sequence of the Mbo I–Mbo I fragment, carried out from the unique Bcl I site, which we mapped to the position shown in Figure 1. We note that the Bcl I site can be seen in the published sequence of DS401 at the same position as in Figure 1, and that its location there is independent of the orientation of the Mbo I–Mbo I fragment. Moreover, we have recently sequenced a portion of a cDNA synthesized from a *var1*[*40.0*] transcript (Zassenhaus et al., manuscript in preparation), and find complete agreement with the orientation and sequence of the Mbo I–Mbo I segment shown here.

Second, our *var1* sequence contains a total of two insertions and four substitutions compared with the [*42.0*] allele given by Tzagoloff et al. (1980); these differences are underlined in Figure 3. Of the latter, two result in amino acid substitutions at positions 92 (Ser to Leu) and 1005 (Ser to Arg) (Figure 3), one is

silent (position 441) and the remaining substitution removes a termination codon (position 436). To resolve these differences, we determined the sequence of a segment of the *var1* region in petite DS401 whose published sequence differs from our [*40.0*] sequence by one insertion and two base changes (at bases 436, 441 and 469 in Figure 3). Our sequence of that segment of DS401 was identical to the sequence in Figure 3. Because of these corrections, we can reconcile the discrepancy between our finding of a continuous reading frame in the *var1*[*40.0*] allele and the apparent absence of such an open reading frame in the [*42.0*] allele sequenced by Tzagoloff et al. (1980).

Third, our sequence lacks two groups of nucleotides whose presence does not shift the reading frame, and that we believe to be allele-specific elements responsible for the size of var1 protein in various strains: one is a 6 base sequence found after nucleotide 489 in Figure 3, and the other is an 18 base sequence found after nucleotide 840 (compare Tzagoloff et al., 1980). These regions are bracketed to indicate that the exact points of insertion are indeterminate. Both inserts are repeats of AAT (Asn) codons and thus lengthen the product of the [*42.0*] allele by a total of 8 asparagine residues compared with that of the [*40.0*] allele. These inserts were called b elements in previous genetic studies of the *var1* region (Strausberg and Butow, 1981). A further analysis of these inserts, in addition to other inserts we have uncovered in different *var1* alleles, will be presented elsewhere (Hudspeth et al., manuscript in preparation).

Discussion

We have identified a long open reading frame in a region of yeast mtDNA previously shown to contain the determinant specifying the size of var1 protein (Strausberg et al., 1978; Vincent et al., 1980). Based on the location of this open reading frame, the close agreement between the amino acid compositions in Table 1 and the agreement between the derived N-terminal sequence and the preliminary amino acid sequence data cited in the Results, we conclude that the open reading frame represents the structural gene for var1 protein. Although we cannot eliminate the possibility of one or more introns within the coding region, the very close agreement between the amino acid composition predicted from the DNA sequence and that found for the protein makes the presence of extensive intervening sequences unlikely.

Additional evidence for the expression of the open reading frame comes from our identification of specific transcripts of this entire sequence and some 1 kb of flanking DNA (Farrelly et al., 1982). In particular, we have found that the putative mRNA for var1 protein, a 16S RNA species, varies in a strain-dependent manner in both size and amount in direct correspondence with the size and amount of var1 protein made. Furthermore, all of the inserts we have identified in the coding region associated with specific *var1* alleles (Hudspeth et al., manuscript in preparation) are found in the 16S *var1* transcript (Zassenhaus et al., manuscript in preparation).

Assignment of the AUA Codon

The agreement between amino acid compositions, and between predicted and preliminary N-terminal amino acid residues, suggests an alteration in the currently used yeast mitochondrial genetic code (Bonitz et al., 1980a) to allow AUA to be read as methionine. This alteration has already been noted for the human (Barrell et al., 1979), bovine (Young and Anderson, 1980) and murine (Bibb et al., 1981) mitochondrial genomes. On yeast mtDNA, other than its occurrence in *var1*, ATA is used a total of seven times in the six protein-coding genes previously sequenced (see Introduction). In the gene encoding cytochrome oxidase subunit I (Bonitz et al., 1980b), the single use of the ATA codon is found in a protein fragment strongly homologous (13 of 17 residues) to its bovine analog, in which that position is occupied by methionine (Figure 5). Methionine is also predicted in the analogous position on human and murine mtDNAs. Of the other six uses of ATA in yeast mitochondrial genes, in five the homologous position in human, bovine or murine mtDNA is occupied by neither methionine nor isoleucine. In the remaining case (in the gene encoding cytochrome oxidase subunit II), the analogous position is in a region of low homology (2 of 17 residues) and is filled by isoleucine on murine and bovine mtDNAs, but by leucine in human mtDNA.

The gene encoding cytochrome oxidase subunit III on Neurospora crassa mtDNA has recently been sequenced (Browning and RajBhandary, 1982). Comparison of the amino acid composition derived from the DNA sequence with that determined for the protein (Sebald et al., 1973) indicates that the AUA codon in Neurospora mitochondria is translated conventionally as isoleucine. In addition, those data support the conventional use of the CUN series for leucine, although our results are consistent with the exceptional use of CUN for threonine in yeast mitochondria (Bonitz

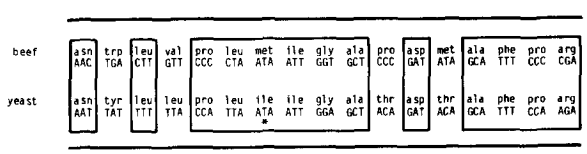

Figure 5. Comparison of Portions of Yeast and Bovine Genes Encoding Cytochrome Oxidase Subunit I

The bovine sequence (Anderson et al., 1982) represents nucleotides 5924–5974. The yeast sequence (Bonitz et al., 1980b) represents nucleotides 5204–5206 and 6721–6769; these sequences are separated by intron sequences. Asterisk: the ATA codon. Boxes: regions of amino acid homology.

et al., 1980a). These comparisons show a startling split in the mitochondrial genetic code among members of the class Ascomycetes. Taken together with the finding that the gene encoding ATPase subunit 9 is on mtDNA in yeast but on nuclear DNA in Neurospora (Sebald et al., 1977; Macino and Tzagoloff, 1979), and that var1 and its cognate protein from Neurospora, S-5, appear unrelated (see below), they suggest that similar organisms have taken parallel but nonidentical paths in the evolution of their mitochondrial genomes.

The recent sequencing of the gene encoding cytochrome oxidase subunit II in Zea mays (Fox and Leaver, 1981) allows the use of ATA in plant mitochondrial genomes to be addressed as well. Unfortunately, the single use of ATA in that gene occurs at a position that in the human protein is occupied by leucine in a region of modest but recognizable homology (6 of 17 residues), and that in bovine (Anderson et al., 1982), murine (Bibb et al., 1981) and yeast (Coruzzi and Tzagoloff, 1979; Fox, 1979) proteins is occupied by leucine, leucine and phenylalanine, respectively. Thus the position specified by ATA in Z. mays is weakly conserved, and is occupied by neither methionine nor isoleucine in the available comparisons.

Proteins Related to var1

Var1 has been shown to be a protein of the small mitochondrial ribosomal subunit, and it has been suggested that it has a role in the assembly of mitochondrial ribosomes (Terpstra and Butow, 1979). Do other mitochondria contain an analogous protein? Neurospora (Lambowitz et al., 1976, 1979), and probably Paramecium (Tait et al., 1976) and Tetrahymena (Perasso et al., 1980), contain a mitochondrially synthesized mitochondrial ribosomal protein. Of these, the Neurospora protein, S-5, has been purified and partially characterized (LaPolla and Lambowitz, 1981). Although the gene for this protein has not been identified, it is presumed to be on Neurospora mtDNA. However, in spite of the similarity in size between S-5 and var1, their amino acid compositions are clearly unrelated. For instance (compare with Table 1), S-5 contains 12.2% aspartic acid plus asparagine, 0.4% methionine, 8.1% alanine and 7.3% glutamic acid plus glutamine (LaPolla and Lambowitz, 1981). One consequence of this difference is that var1 is considerably less polar than S-5 (23% hydrophilic amino acids versus 50%, respectively) and, interestingly in this respect, is more like a "typical" mitochondrial translation product than a ribosomal protein.

Although experiments with inhibitors of mitochondrial protein synthesis implicate both S-5 and var1 in the assembly of mitochondrial ribosomes (LaPolla and Lambowitz, 1977; Lambowitz et al., 1979; Terpstra and Butow, 1979), they may be unrelated and structurally different ribosomal proteins. Possibly, any one of a number of mitochondrial ribosomal proteins, if synthesized in mitochondria, can function equivalently in ribosome assembly.

In this connection, we have searched for homology between the predicted sequence of var1[40.0 kd] protein and that of each of the unidentified reading frames in human mtDNA (Anderson et al., 1981) without finding a match. Since only a minority of the 25 or so mitochondrial translation products detected in HeLa mitochondria (Attardi et al., 1980) have been assigned to known genes, the presence of a mitochondrially translated ribosome-associated protein in higher eucaryotes remains an open question.

If a var1-like protein is necessary for mitochondrial function, we can envision two possible explanations for the lack of homology: one is that var1 is evolving too rapidly for homology in amino acid sequence to be evident by the comparisons we have made, and a second is that yet a different mitochondrial ribosomal protein is translated in human mitochondria. Alternatively, if such a protein need not be translated within mitochondria, then the gene for a var1-like protein in human mitochondria may be nuclear-encoded. The latter explanation is analogous to the observation that the gene for ATPase 9 is present on mtDNA in yeast (Macino and Tzagoloff, 1979) but on nuclear DNA in Neurospora (Sebald et al., 1977).

Codon Usage in *var1*

As seen in Table 2, a number of codons are not used at all, and these are heavily skewed towards codons containing a G or C in the third position in place of an A or U. Bonitz et al. (1980b) first noted that CGN arginine codons have not been found in established genes, but are found in open reading frames, and that phenylalanine is encoded about equally by UUU and UUC in genes, but is strongly biased toward UUU in open reading frames (Table 2). In addition, with our reassignment of the AUA codon, methionine is specified by AUG preponderantly in genes and by AUA preponderantly in open reading frames. Interestingly, *var1* resembles open reading frames rather than genes in all three properties.

The significance of such observations about codon usage is unclear. There seems to be no a priori reason why codon usage should reflect the order in which investigators identify gene products. We can speculate instead that genes with open reading frames represent the past colonization of mitochondria by a different group of genes than those that participate in oxidative phosphorylation. This argument suggests that fungi were colonized at least twice, and that the marked differences in codon usage reflect these independent events.

Organization of the Yeast Mitochondrial Genome

The open reading frame in *var1* is 89.6% AT, the most AT-rich coding region of which we are aware. The

yeast mitochondrial genome is generally considered to be organized as "genes" flanked by AT-rich "spacer DNA" (Bernardi, 1976), the latter comprising some 50% of the total mitochondrial genome. Widely dispersed along the yeast mitochondrial genome are GC-rich clusters (Prunell and Bernardi, 1977), and it has been suggested that these short (30–50 bp) palindromic sequences punctuate genes and serve as sites for RNA processing (Tzagoloff et al., 1980). Our present results, together with the observation that stable *var1* transcripts retain such GC-rich sequences (Zassenhaus et al., manuscript in preparation), require some reevaluation of these models, which assign coding functions only to regions of moderate GC content, and spacer and control functions, respectively, to AT-rich and GC-rich regions. We note that about 30% of the yeast mitochondrial genome (24 kb) has not yet been examined for coding regions because its high AT content appeared to exclude structural genes.

Experimental Procedures

Cells and DNA Preparation

S. cerevisiae petite strain A17-10 was described by Vincent et al. (1980) and obtained from P. S. Perlman. It transmits the *var1[40.0]* allele in crosses with tester strains. Strain DS401 (ρ^-), derived from D273-10B, was used by Tzagoloff et al. (1980) for their sequencing studies; it was obtained from A. Tzagoloff and was shown by us to transmit the *var1[42.0]* allele. Cells were grown to late stationary phase in medium containing 1% yeast extract, 2% peptone, 2% dextrose and harvested by centrifugation. Mitochondrial DNA was isolated as described by Hudspeth et al. (1980).

Labeling of DNA

Restriction enzymes were obtained from New England BioLabs or Bethesda Research Laboratories and were used with the reaction conditions recommended by the manufacturers. We carried out multiple digests sequentially, where possible starting with the enzyme requiring the lowest salt and/or Mg^{2+} concentration, and supplementing the digest before addition of subsequent enzymes. Polynucleotide kinase was obtained from P-L Laboratories or Bethesda Research Laboratories. Alkaline phosphatase (BAPC) was from Worthington. It was dialyzed overnight at 4°C against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA; brought to a concentration of 1 mM Zn^{2+}; heated at 80°C for 20 min; cooled on ice for 5–10 min; and centrifuged for 5 min at 12,800 × g. The supernatant was brought to a concentration of 20%–25% glycerol and stored at −20°C. Escherichia coli DNA polymerase I (large fragment) was obtained from New England Nuclear. γ-^{32}P-ATP (4000–7000 Ci/mmole) was purchased from ICN or Amersham; α-^{32}P-deoxynucleoside triphosphates (3000 Ci/mmole) were from Amersham.

Restriction fragments were labeled at either their 5′ or 3′ ends for DNA sequencing. 5′ labeling was carried out as described by Maxam and Gilbert (1980). In some cases, all restriction cuts with two or more enzymes were made before end labeling to allow the digest to be checked for completion by electrophoresis through 1% agarose (in 40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA [pH 8.2]) or 4% polyacrylamide (in 40 mM Tris base, 20 mM sodium acetate, 2 mM EDTA [pH 7.8]) gels. In these cases, the ends to be labeled were produced with the first enzyme and dephosphorylated. Alkaline phosphatase was removed by deproteinization with phenol, then with phenol plus chloroform (1:1), and the DNA was precipitated with 67% ethanol from a 0.3 M sodium acetate solution. The precipitated DNA was washed and redissolved in the reaction mixture for the next enzyme, and restriction was continued. The final digest was 5′-labeled. With this protocol, ends predicted to be unlabeled were found to be so.

For 3′ labeling, DNA was incubated for 30–45 min at 0°C in 67 mM Tris-HCl (pH 7.4), 7 mM $MgCl_2$, 67 mM NaCl, 200 μM each of unlabeled deoxynucleoside triphosphates and 0.2 mCi labeled triphosphate plus 0.16 U/μg DNA of DNA polymerase I large fragment.

Isolation of End-Labeled DNA Fragments

Fragments were separated after secondary cleavages in 4% polyacrylamide gels. For strand separation, fragments were run through 4%, 5% or 6% polyacrylamide gels in TBE buffer (25 mM Tris-borate, 0.5 mM EDTA [pH 8.3]). For either type of end separation, fragments containing regions of interest were excised and embedded in 0.6% agarose (in TBE buffer plus 1 μg/ml ethidium bromide) on a horizontal gel apparatus. Troughs were cut on one side of the fragment, and the DNA was subjected to electrophoresis into several changes of TBE buffer by 30–60 sec pulses of power at 300 V. Samples containing DNA were pooled and precipitated from 0.1 M ammonium acetate by 2.5 volumes of ethanol. Gel debris was removed by centrifugation, and contaminating acrylamide was removed by serial precipitations from water until the DNA remained in the supernatant. DNA from the final supernatant was precipitated by 2.5 volumes of ethanol after the addition of ammonium acetate to a concentration of 50 mM. For sequencing, the DNA was then resuspended in water.

DNA Sequencing

Sequencing was carried out according to the method of Maxam and Gilbert (1980), with several modifications. Ammonium acetate was used in place of sodium acetate for all precipitations. In addition to the hydrazine reactions for pyrimidine cleavage (and, later, in place of them), the permanganate T and hydroxylamine C reactions (Rubin and Schmid, 1980) were used. Furthermore, a citrate G+A reaction (A. Maxam, personal communication) was carried out in 30 μl by mixing, at 0°C, 16 μl 50 mM citrate (pH 4.0), 0.1 mM EDTA with water and labeled DNA. After incubation for 5 min at 80°C, piperidine release was carried out according to the method of Maxam and Gilbert (1980). Gels were 80 cm long by 0.4 mm thick, and usually contained either 6% or 11% acrylamide. Kodak XR-P film was exposed at −70°C, with Dupont Lightning Plus intensifying screens when necessary.

Purification of var1 Protein

Mitochondria and mitochondrial ribosomal subunits were isolated from strain D′ as previously described (Terpstra and Butow, 1979), with the following modifications: cells were converted to spheroplasts by incubation with 0.3 mg Zymolyase (Miles) per gram (wet weight) of cells; mitochondrial lysis and initial fractionation of ribosomal subunits were carried out according to the procedure of Singh et al. (1978); and 38S ribosomal subunits were isolated from a 15%–30% exponential sucrose gradient following centrifugation in a Beckman SW28 rotor for 12 hr at 28,000 rpm.

The proteins of the small mitochondrial ribosomal subunit were separated on preparative 11% SDS gels (Douglas and Butow, 1976). The gels were stained for 30–45 sec in ice-cold 0.25 M KCl and 1 mM dithiothreitol as described by Hager and Burgess (1980). Strips cut from the gel containing var1 protein were soaked for 20 min at room temperature in 50 mM Tris-Cl (pH 6.8), 2% SDS, 2 mM EDTA and 1% 2-mercaptoethanol. The strips were overlaid on 6.5% SDS gels and subjected to electrophoresis at 12 mA per gel for 16 hr. After staining and excising the var1 band as described above, we soaked the strips for 10 min in 1 mM dithiothreitol. Var1 was eluted by diffusion into 10 mM N-ethylmorpholine acetate (pH 6.8), 0.1% lithium dodecyl sulfate, 2% thiodiglycol and 2 mM dithiothreitol for 48 hr at 37°C, and was concentrated from the elution buffer with an Isco Sample Concentrator (model 1750). Different preparations of purified var1 protein were combined and subjected to electrophoresis a second time through a 6.5% SDS gel, eluted and concentrated as described above. The purified var1 protein was dialyzed against 50 mM ammonium bicarbonate and stored frozen.

Amino Acid Analysis

Samples of var1 protein were hydrolyzed for 15, 24, 48 or 72 hr under reduced pressure in constantly boiling 6 N HCl at 110°C.

Amino acid analyses were performed with an Aminco Aminalyzer. Blanks, baselines and standards were run for each analysis. Except for losses in serine and threonine, the 48 and 72 hr hydrolyses gave essentially the same results as the 15 and 24 hr experiments. A comparison between the predicted and experimentally determined amino acid compositions of var1 to estimate the extent of sequence homology was made with the expression:

$$DT = \tfrac{1}{2}\Sigma|n_{iA} - n_{iB}|$$

(Cornish-Bowden, 1978), where *DT*, the "difference total," represents a quantity that has a minimum value of zero when the amino acid compositions of identical proteins are compared, and n_{iA} and n_{iB} represent the numbers of the *i*th amino acid in protein *A* and protein *B*, respectively.

Two-Dimensional Gel Analysis of Ribosomal Proteins

Proteins were extracted from the small mitochondrial ribosomal subunit after resuspension of the subunits in 30 mM 2-mercaptoethanol, 0.25 M EDTA, 6% thiodiglycol and 1 mM phenylmethane sulfonyl fluoride by addition of 0.1 volume of 1 M $MgCl_2$ and 2 volumes of glacial acetic acid. The RNA precipitate was removed by centrifugation, and the supernatant was desalted on a Sephadex G-25 column equilibrated with 1% acetic acid. Fractions containing the ribosomal proteins were pooled and lyophilized. The first gel dimension was a modification of the Triton–acid urea gel described by Zweidler (1978). The gel, cast in a 0.3 × 10.5 cm tube, contained 8 M urea, 8% polyacrylamide, 0.64% bisacrylamide, 0.37% Triton X-100, 5% acetic acid, 0.5% TEMED and 0.6% $(NH_4)_2S_2O_8$. After pre-electrophoresis of 20 μl of 2 M cystamine in 5% acetic acid, the ribosomal proteins were applied in 10 μl of 8 M urea and 5% acetic acid. Electrophoresis was carried out for 4.5 hr at 140 V. The gel was removed and soaked in 0.5 M Tris (pH 6.8) and 1% SDS for 45 min, and the proteins were subjected to electrophoresis in a second dimension on an 11% SDS gel. Gels were fixed and stained as described by Terpstra et al. (1979).

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