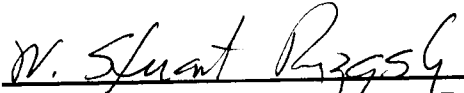
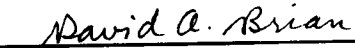


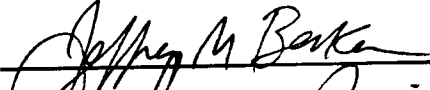
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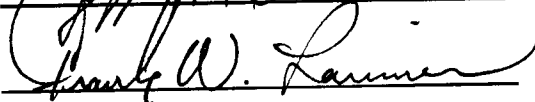
I am submitting herewith a dissertation written by James Andrew Shaw entitled "The Mitochondrial Genome of *Candida albicans*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.


W. Stuart Riggsby, Major Professor

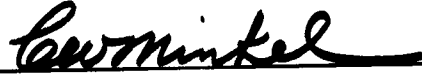
We have read this dissertation
and recommend its acceptance:







Accepted for the Council:



Vice Provost
and Dean of The Graduate School

THE MITOCHONDRIAL GENOME OF
CANDIDA ALBICANS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

James Andrew Shaw
May 1989

This dissertation is dedicated to my mother and my father.

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ABSTRACT

Candida albicans is an opportunistic, pathogenic fungus which is primarily the cause of topical infections of the mucousal surfaces of the mouth and vagina. As an opportunistic pathogen it can also present serious problems for the immunocompromised patient. As part of the routine characterization of this medically important organism and because of the availability of direct methods to locate mitochondrial genes this project was undertaken to study the structure and function of its mitochondrial genome.

The six *Eco*RI fragments of the 40Kb circular mitochondrial (mt) DNA were cloned into the *Eco*RI site of pBR322. A detailed restriction map was prepared for 11 restriction endonucleases. Probes specific for the eight ubiquitous mitochondrial genes (large and small ribosomal RNA's [rRNA], cytochrome oxidase subunits I, II, and III, ATPase subunits 6 and 9, and cytochrome b) were derived from a special class of petite mutants of *Saccharomyces cerevisiae*. These probes were used in Southern blot experiments to detect those mitochondrial restriction fragments having sequence homology with the *S. cerevisiae* gene probes. Expression of the mitochondrial genes was demonstrated by standard Northern blot experiments.

Detailed restriction mapping of the mtDNA of *Candida albicans* confirms the existence of a large inverted duplication. As is the case with some other mt and chloroplast DNAs, the non-duplicated regions of the molecule occur in two orientations with respect to each other, indicating that internal recombination occurs. Like other mtDNA's, the *C. albicans* mtDNA contains a single *Sal*I restriction

site, located near one end of the large rRNA gene. In contrast to other cases however, the inverted duplication does not appear to contain any sequences coding for rRNA.

The eight ubiquitous mitochondrial structural genes mentioned previously have been located on the mtDNA of *Candida albicans*. The relative order of these genes reveals yet another unique arrangement for fungal mitochondrial genomes. The gene order in *C. albicans* is most similar to that of *Saccharomyces exiguus*. All eight genes are expressed and produce transcripts of sizes comparable to the homologous transcripts in other fungi. The genes encoding cytochrome oxidase subunit I, cytochrome b and cytochrome oxidase subunit II appear to have introns.

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PART 1. INTRODUCTION

BACKGROUND AND HISTORY

Description of *Candida albicans*

Candida albicans is a pathogenic fungus with a dimorphic mode of growth. In the yeast phase the single cells reproduce by budding and in the mycelial phase by hyphal extension. This dimorphism is thought to contribute to the pathogenicity of *C. albicans* by conferring upon it an enhanced ability to resist phagocytosis and to invade tissues. However, since several other pathogenic fungi have no mycelial phase the significance of dimorphism as a virulence factor is not clear (6).

Medical importance

The clinical presentation of *Candida albicans* is most commonly an infection of the mucous surfaces of the mouth (oral thrush) and the vagina (vaginal candidosis). However, it can be found infecting a wide range of tissues especially in individuals that are generally unhealthy or undergoing extensive antibiotic therapy. *C. albicans* is a major contributor to morbidity and mortality in immuno-compromised patients (6) and as such has emerged as a serious problem in the care of AIDS patients (1).

Mitochondrial DNA

The study of fungal mitochondrial DNA (mtDNA) has been greatly facilitated by extensive work on the mtDNA of the yeast *Saccharomyces cerevisiae* dating back to the 1940's when Boris Ephrussi discovered that certain respiratory-deficient mutations (called "petite" mutations because of the small colony phenotype they produced) did not segregate by Mendelian rules of inheritance (4). This indicated that the mutation was carried on a cytoplasmic particle that was later shown to be mtDNA. Since then, the mtDNA of *S. cerevisiae* has been thoroughly characterized and almost completely sequenced. The genes present on mtDNA include those for the translational apparatus (large and small ribosomal RNA's and tRNA's), one subunit of the ATPase complex (many fungi have two), the complete apocytochrome b gene, and three subunits of the cytochrome oxidase complex. This set of genes is common to all mtDNA's so far examined from fungi to mammals (7).

The mapping of mitochondrial genes in *S. cerevisiae* was done by a combination of methods (4) all of which rely on the ability to perform sexual crosses. The lack of a sexual system in *Candida albicans* makes such an approach impossible. However, in the course of studying *S. cerevisiae* a special kind of petite mutant was discovered. In such petite mutants the mtDNA molecule has undergone large deletions to the extent that only the sequence for one gene remains (8). The mtDNA from these "single-gene" petites

can be used as probes to locate the homologous genes on physical maps of mtDNA's from other organisms (3). Many fungal mtDNA's (as well as a few protozoan mtDNA's) have now been mapped in this way (3,7).

When the gene maps of fungal mtDNA's are compared to the gene maps of mtDNA's from multicellular organisms two striking differences are observed. Among the fungi the relative order of mitochondrial genes is highly variable from species to species whereas the relative gene order is absolutely constant from *Drosophila* to Man (7). Secondly, the physical size of the mtDNA in fungi ranges from 18Kb to over 100Kb while in animals the size is consistently in the range of 15-18Kb.

There are many reasons to study fungal mtDNA's; foremost among them is to understand their highly variable nature. There may be underlying mechanisms governing this variability which can only be seen when a larger number of fungal mtDNA's have been characterized. Since it has been shown that some closely related fungi have almost identical mitochondrial gene orders (5), previously unidentified taxonomic relationships may be discovered through such studies. This is particularly important for fungi in the catch-all genera of *Candida* and *Torulopsis*. There is also an hypothesis of evolution in mitochondria based on mtDNA size and gene rearrangement (2) which proposes that the ancestral mtDNA was small and evolved by growing larger and becoming rearranged at the same time. Testing of this hypothesis will require the study of many more fungal mitochondrial genomes.

LITERATURE CITED

1. CDC (Centers for Disease Control) Update. 1986. Acquired Immune Deficiency Syndrome—United States. *Morb. Mort. Weekly Rev.* 35: 17.
2. Clark-Walker, G. D., C. R. McArthur and K. S. Sriprakash. 1983. Order and orientation of genic sequences in circular mitochondrial DNAs from *Saccharomyces exiguus*: Implications for evolution of yeast mtDNAs. *J. Mol. Evol.* 19: 333-341.
3. Clark-Walker, G. D., and K. S. Sriprakash. 1981. Sequence rearrangements between mitochondrial DNAs of *Torulopsis glabrata* and *Kloeckera africana* identified by hybridization with six polypeptide encoding regions from *Saccharomyces cerevisiae* mitochondrial DNA. *J. Mol. Biol.* 151: 367-387.
4. Dujon, B. 1981. Mitochondrial genetics and function. in *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance*. (J. N. Strathern, E. W. Jones, J. R. Broach, eds.; New York: Cold Spring Harbor Laboratory) pp. 505-635.
5. Hoebe, P., and G. D. Clark-Walker. (1986). An approach to yeast classification by mapping mitochondrial DNA from *Dekkera/Brettanomyces* and *Eeniella* genera. *Curr. Genet.* 10: 371-379.
6. Odds, F. C. 1979. *Candida and Candidosis*. Leicester University Press.
7. Sederoff, R. 1984. Structural variation in mitochondrial DNA. in *Advances in Genetics*. (J. G. Scandalios, E. W. Caspari, eds.; Academic Press, Inc.). 22: 1-108.
8. Tzagoloff, A. 1982. *Mitochondria*. (New York: Plenum Press).
9. Wallace, D. C. 1982. Structure and evolution of organelle genomes. *Microbiol. Rev.* 46: 208-240.

PART 2. THE INVERTED DUPLICATION IN THE MITOCHONDRIAL
DNA OF *CANDIDA ALBICANS*

SUMMARY

Detailed restriction endonuclease cleavage site mapping of the mitochondrial (mt) DNA of *Candida albicans* confirms the existence of a large inverted duplication. As is the case with some other mt and chloroplast DNAs, the non-duplicated regions of the molecule occur in two orientations with respect to each other, indicating that internal recombination occurs. Again like other mtDNAs, the *C. albicans* mtDNA contains a single *Sa*I restriction site, located near one end of the large ribosomal RNA (rRNA) gene. In contrast to other cases however, the inverted duplication does not appear to contain any sequences coding for rRNA.

METHODS AND MATERIALS

Strains

Candida albicans strain H317 has been described previously (18). *Saccharomyces cerevisiae* strain D273 10B and its single-gene petite mutants DS80, containing the gene for the small rRNA, and DS631, containing the gene for the large rRNA, were identified and provided by Dr. Alexander Tzagoloff, Columbia University.

Enzymes

Restriction endonucleases were purchased from Bethesda Research Laboratories, Rockville, MD, from New England Biolabs, Beverly, MA and, unless otherwise noted, used as recommended by the supplier.

Other materials

Agarose (Seakem ME) was from FMC Bioproducts, Rockland, ME. Nitrocellulose was from Schleicher and Shuell, Keene, NH.

Recombinant DNA

Except when otherwise stated, techniques for restriction endonuclease digestion, electrophoresis, Southern transfer, nick-translation and hybridization were essentially as described by Maniatis et al. (11).

Cloning the E1 fragment

C. albicans H317 mtDNA was isolated as described by Wills et al. (19) and digested with *Eco*RI. Fragments were separated by electrophoresis through a 0.5% agarose gel and the mtDNA fragments were visualized by ethidium bromide staining. An agarose plug containing the E1 fragment was cut from the gel, placed in a dialysis membrane and isolated by electroelution as described by Smith (16). Plasmid pBR322 that had been treated with *Eco*RI and bacterial alkaline phosphatase (11) was mixed with the purified E1 fragment in 10 μ l of ligation buffer (50mM Tris, pH 7.8; 10mM MgCl₂; 20mM dithiothreitol; 10mM ATP and 50 μ g/ml bovine serum albumin (Miles Pentex). The final concentration of each DNA was 5 μ g/ml. Two units of T4 DNA ligase (New England Nuclear) were added and the mixture was incubated for 2 hours at 14°C. To facilitate intramolecular circularization, the reaction mixture was diluted to 200 μ l with fresh ligation buffer and allowed to incubate overnight at 14°C (4).

Transformation of *E. coli* strain DH1 was performed by standard methods (11). Transformants were selected by growth on plates containing 50 μ g/mL ampicillin and 15 μ g/mL tetracycline. Colonies were replicated on a nitrocellulose filter and lysed by the method of Hanahan and Meselson (8), then screened with nick translated mtDNA. Two presumptive positive E1 containing colonies were picked and further characterized.

Mapping of *Ava*I and *Bst*II sites in E2 and E3

2 μ g of each unlabeled plasmid, E2 and E3, were completely digested with *Eco*RI to release the insert from the vector, and then subjected to partial digestion by *Ava*I and *Bst*II in separate reactions. 2 units of enzyme were added to each 20 μ l reaction. 3 μ L aliquots were removed at 2, 5, 10, 15, 30, and 60 minutes and pooled in a microfuge tube on dry ice. The reactions were thawed immediately before loading on a 0.7% agarose gel. After electrophoresis, the gel was blotted to nitrocellulose. Probes specific for the unique ends of each fragment were prepared in the following way. The larger fragments produced by digestion of cloned E2 and E3 by *Ava*I and *Eco*RI (3.9 and 2.3Kb respectively) were isolated by electroelution from agarose gel slices containing the bands. These two fragments comprise the entire small unique region and slightly overlap the ends of the inverted duplication region. The two fragments were mixed and labeled with ³²P by nick translation. The blot described above was probed with this mixture and the resulting autoradiograph allowed the accurate placement of all *Ava*I and *Bst*II sites as a function of their distance from the ends of E2 and E3.

Isolation of mitochondrial DNA from *S. cerevisiae* single gene petites

The two petite strains were grown in 4 liters of YPGal medium (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, Sigma) on a stir plate at room temperature for 48 hours. A wet weight of

40-50 g was obtained. The pellets were washed once with H_2O , repelleted and resuspended in enough H_2O to make a thick slurry. The slurry was vacuum filtered through a Whatman 50 circle.

Cell breakage and mitochondrial DNA isolation were carried out by the method of Garber and Yoder (6) with minor modification. Filtered cells were added to liquid nitrogen in a Waring blender running on high setting. Blending continued for 15 minutes after all cells were added with occasional replenishment of liquid nitrogen. After allowing the liquid nitrogen to boil away, the remaining cell powder was added with rapid-stirring to 400 mL of ice-cold thaw buffer (0.35M Sucrose, 50mM EDTA, 10mM Tris pH 7.4, 20 μ g/mL Proteinase K). When the mixture was completely homogeneous and thawed, it was centrifuged at 3500 rpm in a Sorvall SS-34 rotor at 4°C for 10 minutes. The supernatant was decanted through two layers of cheesecloth, placed in polycarbonate tubes and centrifuged at 30,000 rpm for 30 minutes at 4°C in a Beckman 42.1 rotor. The supernatant was removed by aspiration. The 3500 rpm pellets were resuspended in an additional 400 mL of thaw buffer, using a Dounce homogenizer, and recentrifuged at 3500 rpm for 10 minutes. This supernatant was decanted through cheesecloth as before and placed on top of the previous 30,000 rpm pellets and recentrifuged at 30,000 rpm for 30 minutes. This supernatant was also removed by aspiration.

The pellets were resuspended in 6 mL Lysis buffer (150mM NaCl, 50mM EDTA, 10mM Tris pH 7.4, 20 μ g/mL Proteinase K) with three to five strokes of a Dounce homogenizer. The mixture was brought to 3% SDS to effect organelle lysis, detectable by a slight

clearing and a substantial increase in viscosity. After 10 minutes on ice, the solution was centrifuged in a 15 mL Corex tube at 7000 rpm for 10 minutes. The supernatant was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1).

The solution was brought to 1.68g/mL CsCl (1.3975 refractive index) and 120 μ g/mL Bisbenzimidazole (Hoechst 33258). Ultracentrifugation was performed at 35,000 rpm in a Beckman Ti50 rotor for 48 hours. The uppermost band visible by UV illumination (usually 20-25 mm from the top of the tube) was withdrawn by side puncture. If necessary, purification was accomplished by recentrifugation of the isolated band in a larger volume rotor (Beckman Ti60).

Dye was removed from the isolated bands by repeated extraction against isopropanol saturated with 5M NaCl. CsCl was removed by dialysis and the DNA recovered by ethanol precipitation.

The identity of the isolated DNA was confirmed in the following way. A *Hinc*II/*Hind*III double digest of mitochondrial DNA isolated from *S. cerevisiae* strain D273 10B (wild type parent of DS80 and DS631) was electrophoresed on an agarose gel and transferred to a nitrocellulose filter. Putative petite mitochondrial DNA was labelled with ³²P by nick translation and used to probe the blotted digest of wild type mitochondrial DNA. After washing and autoradiography, signals obtained were compared with the physical and genetic map of D273 10B mitochondrial DNA (5).

Low stringency hybridization conditions

When probing *C. albicans* mtDNA with *S. cerevisiae*-derived probes the conditions of Browning and RajBhandary (1) were used with slight modification. Hybridizations were performed in 10X SSC, 0.05% SDS, 5X Denhardt's solution and 100µg/mL denatured salmon sperm DNA at 45°C for 24 hours. Blots were washed twice in 10X SSC, 0.05% SDS at 45°C for 30 minutes, once in 2X SSC, 0.05% SDS at room temperature for 30 minutes and once in 1X SSC, 0.05% SDS at room temperature for 30 minutes.

RESULTS

Cloning fragment E1

The putative positive E1-containing plasmids described in Materials and Methods were cleaved with *EcoRI*, and electrophoresed on 1% agarose gels. The gels were stained with ethidium bromide and photographed under ultraviolet illumination. The DNA was transferred to nitrocellulose by the method of Southern (17), and then hybridized to nick-translated ³²P-labeled mtDNA by standard laboratory methods. The identity of the E1 fragment was confirmed by comigration of the cloned fragment with the E1 fragment from whole mtDNA, by the hybridization signal produced, and by the identity of internal restriction sites in the E1 region of whole mtDNA and in the cloned fragment (data not shown).

Detailed restriction map

Mapping of the restriction sites within each cloned fragment was carried out for the most part using a combination of single and double limit digests. However, it was not possible to place all of the *AvaI* and *BstEII* sites in fragments E2 and E3 by the digests just described. In order to determine these sites, the fragments were analyzed by the modified end-labeling technique described in METHODS. The resulting map is shown as Figure 1. (In an earlier report (19), the *SalI* site at 8.3Kb on fragment E2 was incorrectly labeled as a *SmaI* site.)

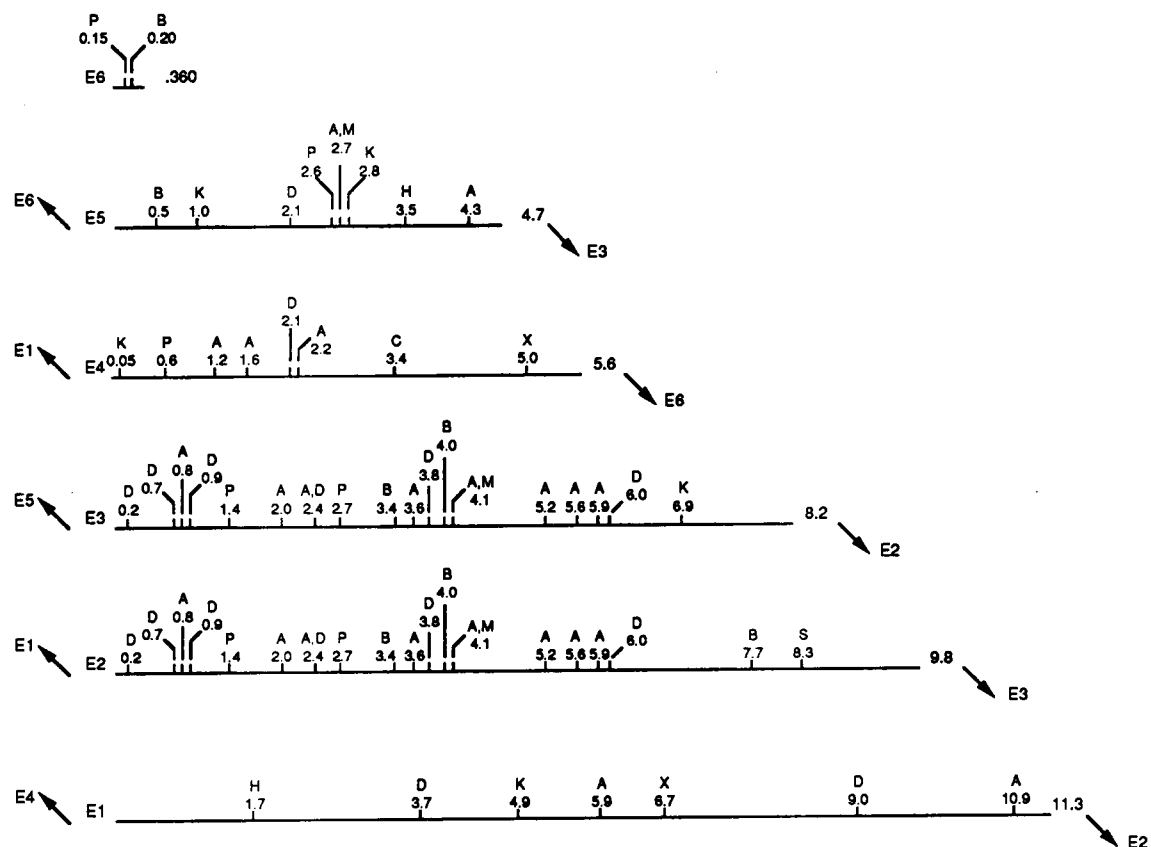


Figure 1: Restriction map of the cloned fragments of *C. albicans* mtDNA. Arrows at the termini of each fragment are included to indicate the adjacent fragment in the whole molecule. The designations for the enzymes are: A - *Ava*I, B - *Bam*HI, C - *Cla*I, D - *Bst*EII, E - *Eco*RI, H - *Hind*III, K - *Kpn*I, M - *Sma*I, P - *Pvu*II, S - *Sal*I, X - *Xba*I

Internal rearrangement

We have shown before (19) that the *C. albicans* mtDNA contains a large inverted duplication, illustrated by the heavy pointed arcs in Figure 2. This duplication is confirmed by the detailed restriction map shown in Figure 1. Figure 1 also shows the *KpnI* restriction sites deduced from the mapping data of Figure 1. It is to be noted that there are five *KpnI* recognition sites, all of them in the non-duplicated parts of the molecule. Consequently, digestion of whole mtDNA with *KpnI* should yield five fragments (designated K1-K5), whose sizes should be 18.5, 8.8, 6.9, 5.0, and 1.8Kb respectively. The results of such a digestion are shown in Figure 3. There are two unusual aspects to this result. First, in addition to the five expected bands, there is an additional band representing fragments of about 13.6Kb. Second, the intensities of the bands do not decrease with decreasing size as expected. In particular, the novel 13.6Kb fragment has a signal more intense than that of the 18.5Kb fragment. These results can be explained if the molecule undergoes internal homologous recombination, as has been observed in some other organelle genomes (9, 12). This phenomenon is illustrated in Figure 4.

Location of ribosomal RNA genes

Inverted duplications have been found in the mitochondrial DNA's of some other fungi (2, 9) and all of these duplications contain sequences coding for ribosomal RNA. To determine the location of

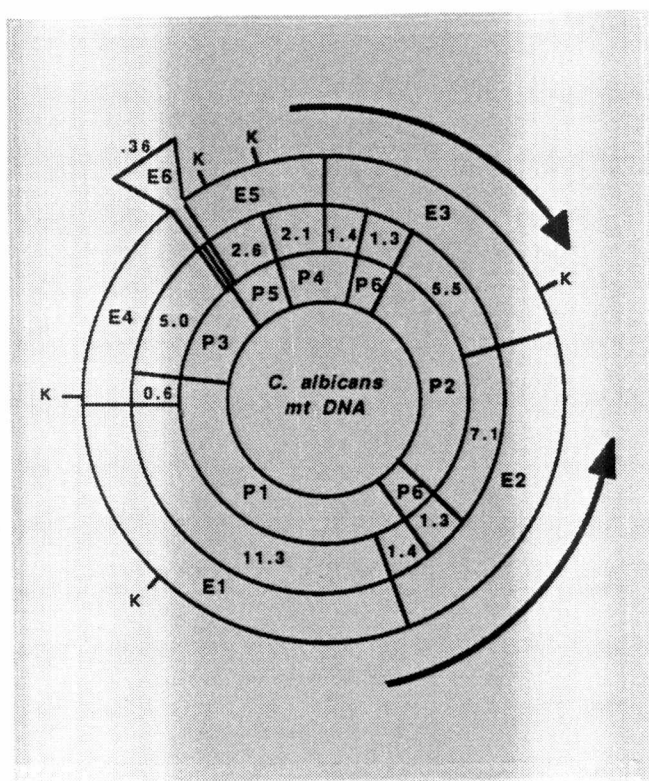


Figure 2: Circular restriction map of *C. albicans* mtDNA. *Eco*RI and *Pvu*II restriction sites are indicated within the circles. *Kpn*I restriction sites determined from the cloned fragments are indicated on the periphery. The heavy arcs indicate the approximate position of the inverted duplication.

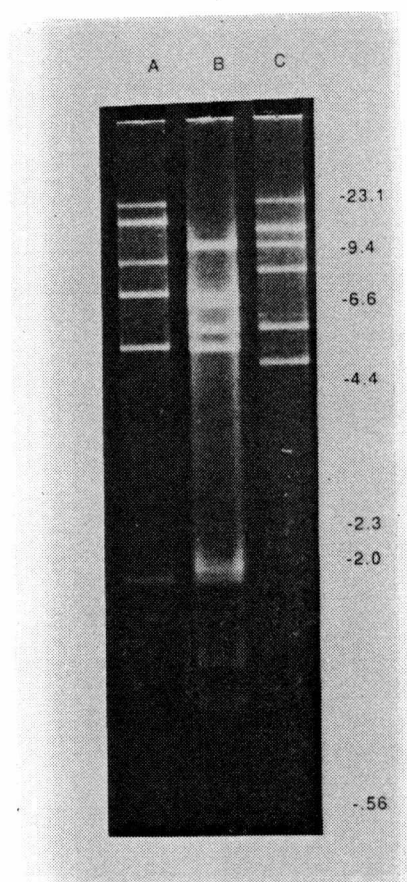


Figure 3: Restriction fragment analysis of *C. albicans* mtDNA with *KpnI*. Lane A contains whole mtDNA digested with *KpnI*. Lane B contains whole mtDNA digested with both *EcoRI* and *KpnI*. Lane C contains whole mtDNA incompletely digested with *EcoRI*. The sizes of marker fragments (phage lambda DNA digested with *HindIII*) are shown on the right.

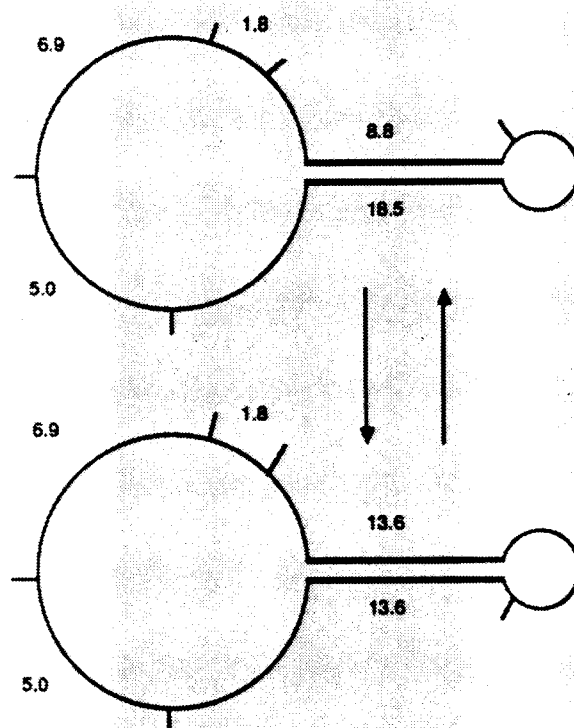


Figure 4: Generation of unexpected *KpnI* restriction fragments by internal homologous recombination of *C. albicans* mtDNA. The structure in the upper part of the figure is a redrawing of the *KpnI* map shown in Figure 2. Here the inverted duplication is indicated by the two linear horizontal lines, and the non-duplicated regions by the circular bubbles. *KpnI* sites are indicated by the ticks, and the size of the expected fragments are indicated. Homologous recombination within the duplication would produce the structure shown in the lower part of the figure.

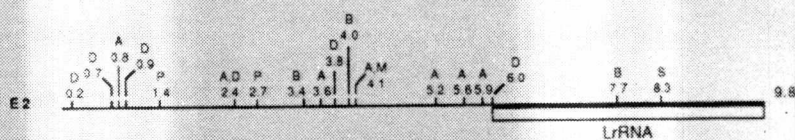
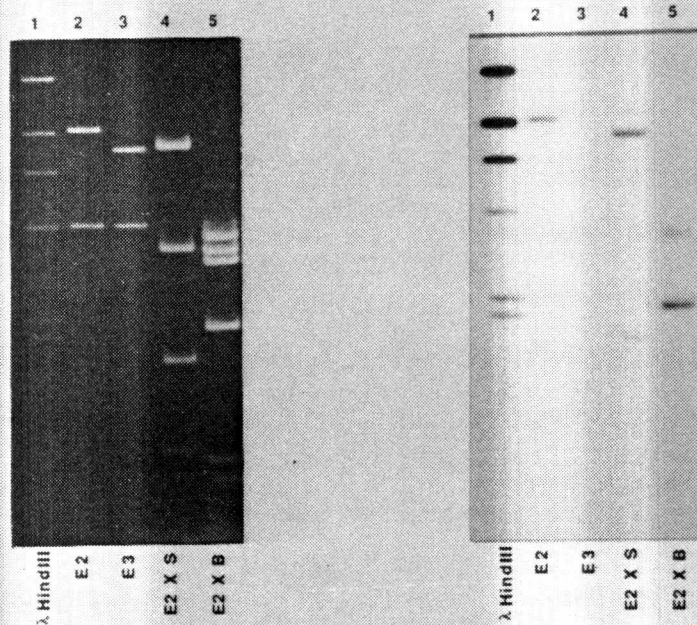
the rRNA gene in the *C. albicans* mitochondrial genome, each of the plasmids containing the *EcoRI* fragments was digested with *EcoRI*, and these fragments were electrophoresed on 1% agarose gels and transferred to nitrocellulose. The blots were probed with ³²P-labeled nick-translated mtDNA from either strain DS631 for the large rRNA or strain DS80 for the small rRNA. This allowed for the primary localization of sequence homology to one of the six *EcoRI* fragments. The fragment giving a positive signal was then further digested with the appropriate enzymes and probed in the manner described above. The results of these experiments are shown in Figure 5 and described below.

Location of the large ribosomal RNA gene

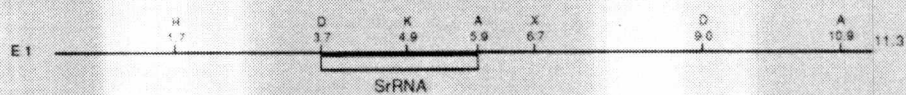
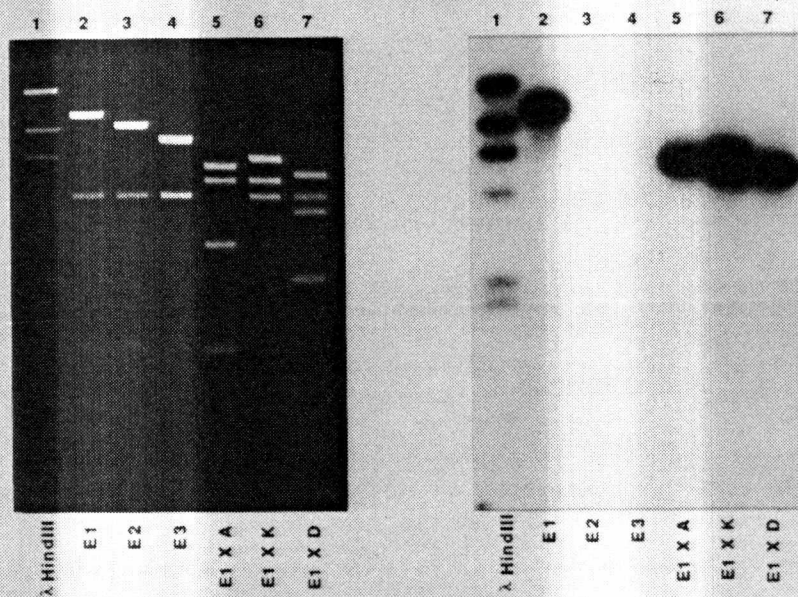
Probing the six *EcoRI* fragments with DS631 mtDNA (large rRNA probe) yielded a solitary signal from fragment E2 (Fig. 5A, lane 2). Since the inverted duplication is present on E2 and E3, the lack of a signal from E3, lane 3, implies that the gene lies entirely within the unique portion of E2. This was confirmed by probing E2 digested with *BstEII* (data not shown). *SaII* and *BamHI* digests (lanes 4 and 5 respectively) yield signals from two fragments each, both of which contain sequences from the unique portion of E2. We can conclude that one end point of the large rRNA gene lies between the *BstEII* site at 6.0Kb and the *BamHI* site at 7.7Kb and the other is to the right of the *SaII* site at 8.3Kb. Based on the intensity of signals from the *BamHI* and *SaII* digests, it is likely that the latter endpoint is closer to the *SaII* site than to the end of the fragment.

Figure 5: Location of ribosomal RNA genes of *Candida albicans* mitochondrial DNA. A. Ethidium bromide stained gel and autoradiograph of blot probed with ³²P-labelled mtDNA from petite strain DS631. Lane 1, lambda *Hind*III marker; lane 2, fragment E2 digested by *Eco*RI; lane 3, fragment E3 digested by *Eco*RI; lane 4, fragment E2 digested by *Eco*RI + *Sal*I; lane 5, fragment E2 digested by *Eco*RI + *Bam*HI. (Fragments E1 and E4-6, which gave no signals, are not included here for the sake of clarity). B. Ethidium bromide stained gel and autoradiograph of blot probed with ³²-P labeled mtDNA from petite strain DS80. Lane 1, lambda *Hind*III marker; lane 2, fragment E1 digested by *Eco*RI; lane 3, fragment E2 digested by *Eco*RI; lane 4, fragment E3 digested by *Eco*RI; lane 5, fragment E1 digested by *Eco*RI + *Ava*I; lane 6; fragment E1 digested by *Eco*RI and *Kpn*I; lane 7, fragment E1 digested by *Eco*RI + *Bst*EII. (Fragments E4-E6, which gave no signals, are not included here for the sake of clarity).

A



B



Clark-Walker et al. (3) observed that in most fungal mitochondrial DNA's a single *Sa*I site near one end of the large ribosomal RNA gene is strongly conserved. It was demonstrated that this site was near the 3' end of the gene in both *Torulopsis glabrata* and *Kloeckera africana* (2). This was also shown to be the case in *Saccharomyces cerevisiae* (5) and in *Achlya ambisexualis* (15). Therefore it appears that this *Sa*I site is a reliable indicator of the polarity of the large ribosomal RNA gene. We have demonstrated the existence in *C. albicans* of a single *Sa*I site near one end of the large ribosomal RNA gene (Fig. 5A, lane 4). Therefore, we can infer the following orientation of the gene: The 5' end is proximal to the end of the inverted repeat in fragment E2 and the 3' end is proximal to the *Eco*RI site at the junction of fragments E2 and E3.

Location of the small ribosomal RNA gene

Probing the six *Eco*RI fragments with DS80 mtDNA (small rRNA probe) yielded a solitary signal from fragment E1 and no signal from fragments E2 and E3 (Fig. 5B, lanes 2, 3, and 4). Therefore the small rRNA gene also does not lie within the inverted duplication. Digestion of E1 with *Ava*I, lane 5, yields a solitary signal from the 5.9Kb fragment. Digestion with *Kpn*I, lane 6, yields signals from both the 4.9Kb and the 6.4Kb fragments. Digestion with *Bst*EII, lane 7, yields a solitary signal from the 7.6Kb fragment. This narrows down the location of the small rRNA gene to the 2.2Kb *Bst*EII/*Ava*I fragment of E1. This places the gene at least 5Kb away from the end of the inverted duplication.

DISCUSSION

We have previously reported the cloning of five of the six *EcoRI* restriction fragments by ligating a mixture of pBR322 DNA and whole mtDNA from *C. albicans* strain SR1010, both cleaved with *EcoRI* (19). Using this procedure, we were unable to obtain a clone containing the largest fragment, E1. Consequently, we cloned this fragment by the alternative procedure described here.

Our analysis and interpretation of the internal recombination in the inverted duplication follows the strategy of Hudspeth et al. (9) and of Palmer (12), whose Figure 3 is a model for our Figure 4. In order to detect this phenomenon, it is necessary to use an enzyme which has cleavage sites only in the non-duplicated part of the molecule. Inspection of the map shown in Fig. 2 shows that *KpnI* satisfies this requirement for *C. albicans* mtDNA. Generally, an internal recombination event leads to the production of two unexpected bands. However, the relative positions of the relevant sites may be such that the two new fragments are not resolvable (9); that is the case here, so that we observe a single band of double intensity, rather than two separate bands.

Superficially, it might appear that the results obtained with *KpnI* digestion could be attributed to incomplete digestion of the DNA, rather than to the appearance of additional fragments due to internal recombination. On closer analysis, however, it is clear that this cannot be the case. First, the additional fragments generated are smaller than the largest band predicted by the data from the cloned fragments; incomplete digestion would be expected to

produce larger, rather than smaller fragments. Second, no combination of two adjacent fragments would give a fragment of the observed size: the sum of K3 and K4 is 11.9Kb, which is about 2Kb smaller than the unexpected fragment. The sum of K3, K4, and K5 is 13.7Kb, which is approximately the size of the unexpected fragment; to account for this fragment by incomplete digestion, it would be necessary for three of the five sites to experience incomplete digestion. But even this strained interpretation is ruled out by the relative intensities of the signals. If the unexpected fragment is an incompletely digested fragment K3 + K4 + K5, then its appearance would be at the expense of signals from those three separate fragments. As the data show, however, it is fragments K1 and K2 whose intensities are reduced relative to the other bands (Fig. 3), not K3, K4, and K5. Finally, the pattern shown in Figure 3 is observed when the DNA is digested for 40 hours at a ten-fold excess of enzyme (data not shown). We conclude that incomplete digestion cannot account for the unexpected fragment, and that internal recombination does occur in this DNA.

The discovery of an inverted duplication in *Candida albicans* mitochondrial DNA marks only the third such instance in fungal mitochondrial genomes. The mtDNAs of the yeast *Kloeckera africana* (2) and the oomycete *Achlya ambisexualis* (9) also contain inverted duplications. The only other mtDNA known to have an inverted duplication is the linear molecule found in the protozoan *Tetrahymena* (7). In contrast to this, the inverted duplication is

practically a standard feature of chloroplast DNAs. Only a few species of legumes have been found that lack inverted duplications in their chloroplast DNAs (13).

In all organelle DNAs that contain an inverted duplication, the size of the duplicated region appears to vary with the size of the entire molecule in a regular way. Although the size of the complete molecule varies from 27Kb to 145Kb, the fraction that is duplicated is consistently in the range of 30 to 39% (2, 10, 13) *Candida albicans* is no exception to this generalization. This consistency may arise through a compromise between the supposed stability gained from an inverted duplication and the energy required to replicate the extra DNA. Palmer et al. (14) point out a strong correlation between the presence of an inverted repeat and a stable chloroplast genome. However, they also showed that deletion of an inverted duplication did not always result in an unstable genome. In an analysis of the *Achlya* inverted repeat, Shumard et al. (15) concluded that this stability is limited to the portion that encodes the ribosomal RNA genes.

Until now all organelle DNAs with inverted duplications were observed to carry all or part of the ribosomal sequences within the duplicated region. In *Kloeckera* only a portion of the large rRNA gene is in the duplication; in *Tetrahymena* the entire large rRNA is in the duplication while the small rRNA is not. In *Achlya* both rRNA genes are in the inverted repeat, and in chloroplasts (which carry an additional, smaller rRNA gene) all the rRNA genes are in the duplication. This consistency raises the question of whether an inverted duplication itself can confer stability upon the molecule

containing it, or whether specific coding sequences within the duplication (in particular, rRNA genes) are also necessary. The fact that no rRNA sequences are present in the inverted duplication in *C. albicans* makes it potentially useful as a model system for studying this phenomenon.

LITERATURE CITED

1. Browning, K. S., and U. L. RajBhandary. 1982. Cytochrome oxidase subunit III gene in *Neurospora crassa* mitochondria. J. Biol. Chem. 257:5253-5256.
2. Clark-Walker, G. D., and K. S. Sriprakash. 1981. Sequence rearrangements between mitochondrial DNAs of *Torulopsis glabrata* and *Kloeckera africana* identified by hybridization with six polypeptide encoding regions from *Saccharomyces cerevisiae* mitochondrial DNA. J. Mol. Biol. 151:367-387.
3. Clark-Walker, G. D., C. R. McArthur, and K.S. Sriprakash. 1983. Order and orientation of genic sequences in circular mitochondrial DNAs from *Saccharomyces exiguus*: Implications for evolution of yeast mtDNAs. J. Mol. Evol. 19:333-341.
4. Dugaiczyk, A., H. W. Boyer, and H. M. Goodman. 1975. Ligation of *Eco*RI endonuclease-generated DNA fragments into linear and circular structures. J. Mol. Biol. 96:171-184.
5. Dujon, B. 1981. Mitochondrial genetics and function. In *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance*. (J. N. Strathern, E. W. Jones, J. R. Broach, eds.; New York: Cold Spring Harbor Laboratory) pp. 505-635.
6. Garber, R. C. and O. C. Yoder. 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. Anal. Biochem. 135:416-422.
7. Goldbach, R. W., P. Borst, J. E. Bollen-deBoer, and E. F. J. Van Bruggen. 1978. The organization of ribosomal RNA genes in the mitochondrial DNA of *Tetrahymena pyriformis* strain ST. Biochim. Biophys. Acta. 521:169-186.
8. Hanahan, D., and M. Meselson. 1980. Plasmid screening at high colony density. Gene. 10:63-67.

9. Hudspeth, M. E. S., D. S. Shumard, C. J. R. Bradford, and L. I. Grossman. 1983. Organization of *Achyla* mtDNA: A population with two orientations and a large inverted repeat containing the rRNA genes. *Proc. Natl. Acad. Sci.* 80:142-146.
10. Kolodner, R. and K. K. Tewari. 1979. Inverted repeats in chloroplast DNA from higher plants. *Proc. Natl. Acad. Sci. U.S.* 76:41-45.
11. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
12. Palmer, J. D. 1983. Chloroplast DNA exists in two orientations. *Nature* 301:92-93.
13. Palmer, J. D., and W. F. Thompson. 1982. Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. *Cell* 29:537-550.
14. Palmer, J. D., B. Osorio, J. Aldrich, and W. F. Thompson. 1987. Chloroplast DNA evolution among legumes: Loss of a large inverted repeat occurred prior to other sequence rearrangements. *Curr. Genet.* 11:275-286.
15. Shumard, D. S., L. I. Grossman, and M. E. S. Hudspeth. 1986. *Achyla* mitochondrial DNA: gene localization and analysis of inverted repeats. *Mol. Gen. Genet.* 202:16-23.
16. Smith, H. O. 1980. Recovery of DNA from gels. *Methods Enzymol.* 65:371-380.
17. Southern, E. 1979. Gel electrophoresis of restriction fragments. *Methods Enzymol.* 68:152-176.
18. Torres Bauza, L. J., and W. S. Riggsby. 1980. Protoplasts from yeast and mycelial forms of *Candida albicans*. *J. Gen. Microbiol.* 119:341-349.

19. Wills, J. W., W. B. Troutman, and W. S. Riggsby. 1985. Circular mitochondrial genome of *Candida albicans* contains a large inverted duplication. J. Bacteriol. 164:7-13.

PART 3. STRUCTURE AND EXPRESSION OF THE MITOCHONDRIAL
GENOME OF *CANDIDA ALBICANS*

SUMMARY

Eight mitochondrial structural genes encoding the large and small ribosomal RNA's, cytochrome oxidase subunits I, II, and III, ATPase subunits 6 and 9, and cytochrome b have been located on the 40 kilobase mitochondrial DNA of *Candida albicans*. The relative order of these genes reveals yet another unique arrangement for fungal mitochondrial genomes. The gene order in *C. albicans* is most similar to that of *Saccharomyces exiguus*. Only two changes (a duplication and an inversion) of the *S. exiguus* order are necessary to attain the order observed in *C. albicans*. All eight genes are expressed and produce transcripts of sizes comparable to the homologous transcripts in other fungi. The genes encoding cytochrome oxidase subunit I, cytochrome b and cytochrome oxidase subunit II appear to have introns since multiple transcripts are observed for these genes.

MATERIALS AND METHODS

Strains and plasmids

Candida albicans strain H317 has been described previously (18).

The *Saccharomyces cerevisiae* single-gene petite strains described in Table 1 were provided by Alexander Tzagoloff, Columbia University. Plasmids pE1-pE6 refer to *C. albicans* mtDNA *EcoRI* fragments E1-E6, respectively, cloned into the *EcoRI* site of pBR322(19,21).

Enzymes

Restriction endonucleases were purchased from Bethesda Research Laboratories, Rockville, MD, and from New England Biolabs, Beverly, MA. Unless otherwise specified enzymes were used according to the supplier's instructions.

Other materials

Agarose (Seakem GTG) was from FMC Bioproducts, Rockland ME. Transfer of nucleic acids to membrane filters was done with both Nitrocellulose (BA85, Schleicher and Schuell, Keene, NH) and Biotrans Nylon Membranes (ICN, Irvine, CA). Northern blots utilized only the nylon membranes.

Table 1. *Saccharomyces cerevisiae* single-gene petite strains

Strain	Gene carried on mtDNA	Reference
DS302	COII (Cytochrome oxidase subunit 2)	(8)
DS31	COIII (Cytochrome oxidase subunit 3)	(17)
DS6 A422	COI (Cytochrome oxidase subunit 1)	(1)
DS14	ATP6 (ATPase subunit 6)	(12)
DS400/A3	ATP9 (ATPase subunit 9)	(11)
DS400/M4	exons 1 and 2 of cytochrome b	(16)
DS400/M11	exon 3 of cytochrome b	(16)

Formamide (Spectro Grade from Kodak, Rochester, NY) was deionized before use by mixing with a mixed bed resin, AG501-X8(D) from BioRad, Richmond, CA. Diethylpyrocarbonate (DEPC) was from Sigma.

Recombinant DNA

Except when otherwise stated, techniques for restriction endonuclease digestion, electrophoresis, Southern transfer, nick-translation and hybridization were essentially as described by Maniatis et al. (13).

Isolation of mitochondrial DNA from *S. cerevisiae* single-gene petites

This procedure was described in detail previously(see part 2).

Preparation of *Candida albicans* gene-specific probes

For Northern blot experiments it was necessary to use probes derived from *C. albicans* mtDNA rather than the *S. cerevisiae* single-gene petite probes. Based on the mapping data, restriction fragments carrying all or part of each *C. albicans* mitochondrial gene were isolated by electroelution from agarose gel slices. In most cases the ease with which a particular fragment could be isolated away from other fragments produced by the digest dictated the choice of which fragment was used. Therefore most of the probes contain flanking sequences and do not carry the entire gene.

Table 2 shows the derivation of each probe. Probes were labeled with ^{32}P with an oligolabeling kit from Pharmacia.

Isolation of mitochondrial RNA from *Candida albicans*

Cells were grown to late exponential phase in 4 L of YPG medium (1% Bacto Yeast Extract, 2% Bacto Peptone, 3% Glycerol) on a stir plate at room temperature. A wet weight of 20-25g was obtained. The pellets were washed once with H_2O , repelleted and resuspended in enough H_2O to make a thick slurry. The slurry was vacuum filtered through a Whatman 50 circle.

The filtered cells were mechanically ruptured by blending with liquid nitrogen and resuspended in high osmolarity buffer (the procedure to this point is identical to that for isolation of mtDNA from petites described previously (see Part 2 pg. 10)). For all steps beyond this point glassware was baked and buffers were made with DEPC-treated water.

The thawed and resuspended cell preparation was centrifuged at 30,000 rpm for 30 minutes in a Beckman 42.1 rotor. The pellet was resuspended in 10mL of 4M guanidine thiocyanate, 50mM tris-HCL pH 7.6, 10mM EDTA, 2% sarkosyl and 140mM 2-mercaptoethanol (6). The remainder of the procedure is exactly as described by Clark-Walker and Sriprakash (6). RNA was obtained at a concentration of about $1\mu\text{g/mL}$ as determined by spectrophotometry.

Table 2. *Candida albicans* gene-specific DNA probes.

Gene	Restriction Fragment	Length (Kb)
ATP6	E5 digested by <i>KpnI</i> + <i>HindIII</i>	0.7
ATP9	E5 digested by <i>HindIII</i> + <i>EcoRI</i>	1.2
COI	E4 entire fragment	5.6
COII	E3 digested by <i>KpnI</i>	1.3
COIII	E3 digested by <i>BamHI</i>	3.4
Cyt. b	E1 digested by <i>HindIII</i>	1.7
Large rRNA	E2 digested by <i>SalI</i> + <i>SmaI</i>	4.2
Small rRNA	E1 digested by <i>KpnI</i> + <i>XbaI</i>	1.8

RNA analysis

RNA (1-2 μ g) was denatured in the presence of formamide at 55°C and loaded on a 1% agarose/2.2M formaldehyde gel. Electrophoresis was carried out on a horizontal submarine gel apparatus (Hoeffer) at 20 V for 18 hours. The gel lane containing the RNA size markers (BRL) was cut off, stained with ethidium bromide and photographed. The remainder of the gel was set up for a Northern blot as described by Maniatis, et al. (13). The Northern blot was cut into strips and each strip probed separately with the *Candida albicans* gene-specific probes described earlier. Hybridization and subsequent washes under high stringency conditions (Hybridization-6X SSC, 0.01M EDTA, 5X Denhardt's solution, 0.5% SDS, 100 μ g/mL Salmon sperm DNA, at 68°C. Washes-twice for 15 min. in 2X SSC, 0.1% SDS at room temp., 3 times for 30 min. in 0.1X SSC, 0.1% SDS at 50°C) as well as autoradiography were as described by Maniatis et al. (13).

RESULTS

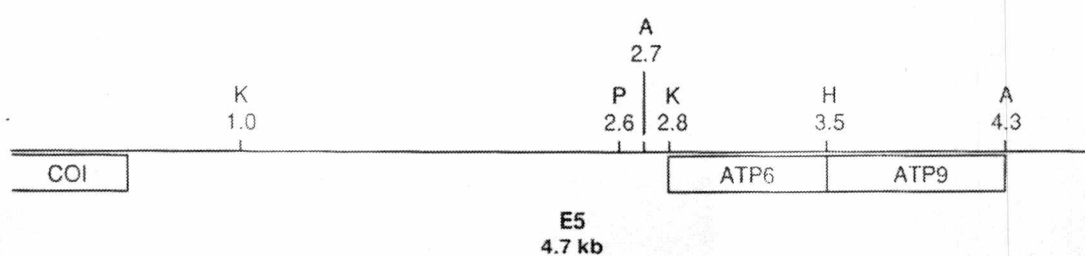
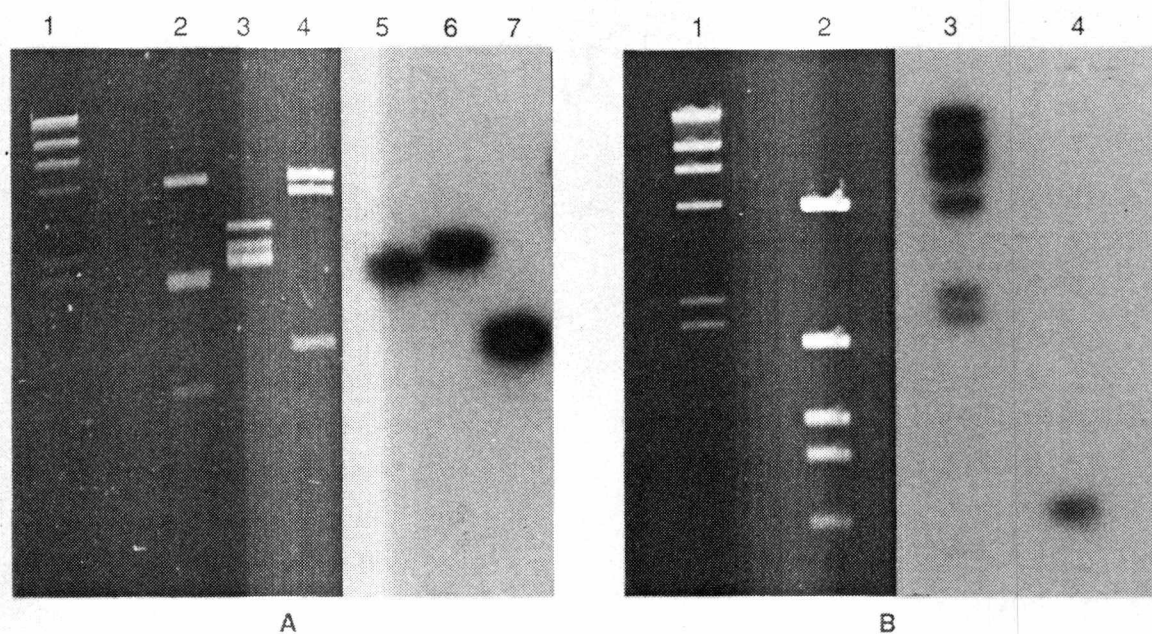
Locations of the genes encoding ATPase subunits 6 and 9

mtDNAs from *S. cerevisiae* petite strains DS14 for ATPase subunit 6 and DS400 A3 for subunit 9 were labeled with ^{32}P by nick-translation and used to probe Southern blots of the six *EcoRI* fragments of *Candida albicans* mtDNA. Autoradiographic signals from both probes were limited to the 4.7Kb fragment E5 (data not shown). Panel A of Figure 1 shows the results of detailed Southern blot analysis using the DS400 A3 (ATPase subunit 9) probe. In lane A4, pE5 was cut with *EcoRI* and *HindIII* yielding a 3.5Kb and a 1.2Kb fragment as well as the vector band of 4.4Kb. The autoradiograph shows in lane 7A that hybridization was limited to the 1.2Kb *HindIII/EcoRI* fragment. Subsequent analysis using *AvaI* indicated homology to the ATPase subunit 9 probe does not extend beyond the *AvaI* site at 4.3Kb (data not shown). This *AvaI* site marks the outside limit of the inverted duplication and its partner is found on the E2 proximal end of E1. Had any hybridization been found on the right side of this site in E5, presumably it would have also been observed on E1. Therefore it is likely that ATPase subunit 9 sequences do not extend into the inverted duplication.

Detailed Southern blot analysis using the DS14 (ATPase 6) probe is shown in Panel B of Figure 1. pE5 was digested with *EcoRI*, *KpnI* and *HindIII* (lane B2). This yielded a 4.4Kb vector band plus E5-derived bands of 1.8, 1.2, 1.0 and 0.7Kb.

Figure 1. Location of ATPase subunits 6 and 9.

pE5 was digested with the indicated restriction endonucleases, electrophoresed in 1% agarose gels and Southern blotted. A- Gel and autoradiograph of blot probed with labeled mtDNA from *S. cerevisiae* petite strain DS400 A3 (ATPase subunit 9). Lane 1, λ HindIII size marker; lane 2, E5 digested with *EcoRI* and *KpnI*; lane 3, E5 digested with *EcoRI* and *PvuII*; lane 4, E5 digested with *EcoRI* and *HindIII*; lanes 5, 6 and 7, autoradiograph of lanes 2, 3 and 4, respectively. B- Gel and autoradiograph of blot probed with labeled mtDNA from petite strain DS14 (ATPase subunit 6). Lane 1, λ HindIII size marker; lane 2, E5 digested with *EcoRI*, *KpnI* and *HindIII*; lane lanes 3 and 4, autoradiograph of lanes 1 and 2 respectively. Diagram at bottom shows interpretation of results. Symbols above line designate restriction endonuclease recognition sites and their distances in Kb from the left end of the fragment. Some sites have been omitted for clarity. A-AvaI, B-BamHI, C-ClaI, D-BstEII, E-EcoRI, H-HindIII, K-KpnI, M-SmaI, P-PvuII, S-SalI, X-XbaI.



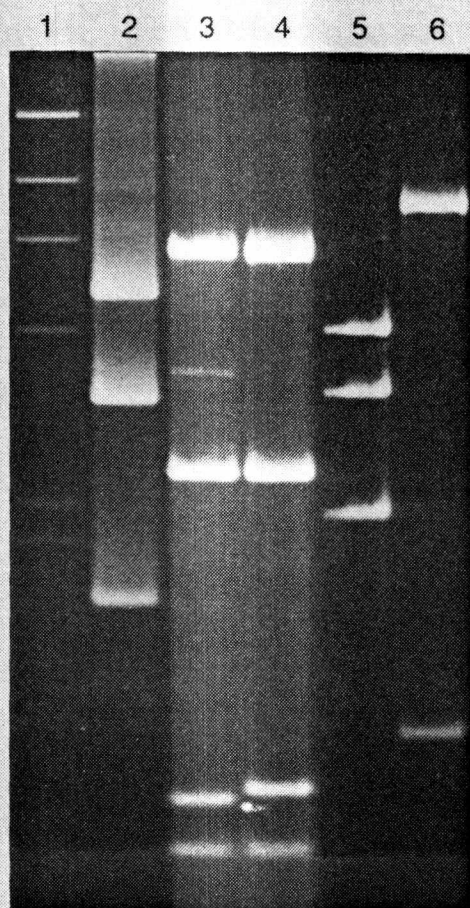
The autoradiograph shows that the probe hybridized only with the 0.7Kb *KpnI/HindIII* As shown in the diagram at the bottom of the figure, this places the ATPase subunit 6 gene immediately to the left of the ATPase subunit 9 gene.

Locations of the genes encoding Cytochrome oxidase subunits I, II, and III

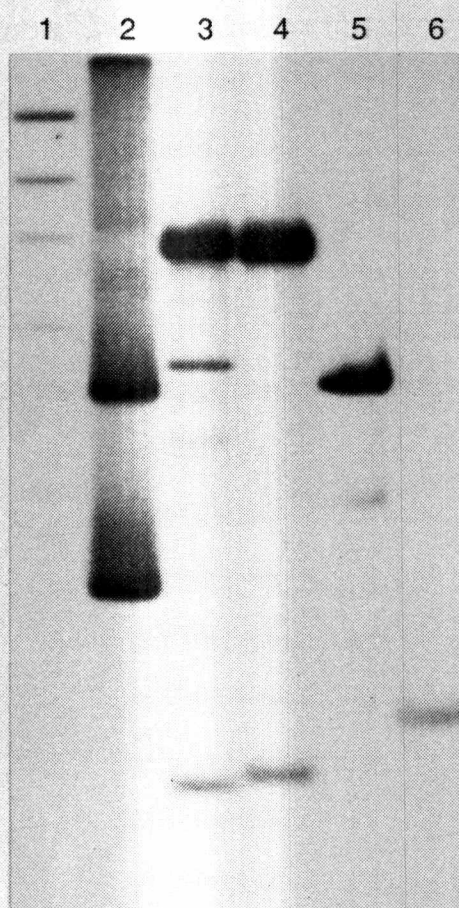
When the six *C. albicans* *EcoRI* fragments were probed with mtDNA from the petite strain DS6 A422 (Cytochrome oxidase subunit I or COI) hybridization was observed from fragments E4, E5 and E6 (data not shown). In the intact mtDNA molecule, these 3 fragments are adjacent to each other with E6 lying between E4 and E5. Therefore it appears that E6 consists entirely of COI sequences. Detailed Southern blot analysis is shown in Figure 2. In lane A2 pE4 was digested with *ClaI* and *XbaI* producing three fragments: one internal *ClaI/XbaI* fragment of 1.6Kb and two insert/vector junction fragments. The largest fragment (approximately 4.9Kb) contains the 0.6Kb *XbaI/EcoRI* fragment of E4 plus 4.3Kb of pBR322 DNA. There is no autoradiographic signal from this band (lane B2). This lack of homology with the probe is indicated by the shaded portion of the gene in the diagram at the bottom of the figure. This fragment lies at the E6 proximal end of E4 and, since the COI probe was observed to hybridize with E6 and E5, it is possible that the fragment contains intron sequences (see discussion).

Figure 2. Location of Cytochrome oxidase subunit I.

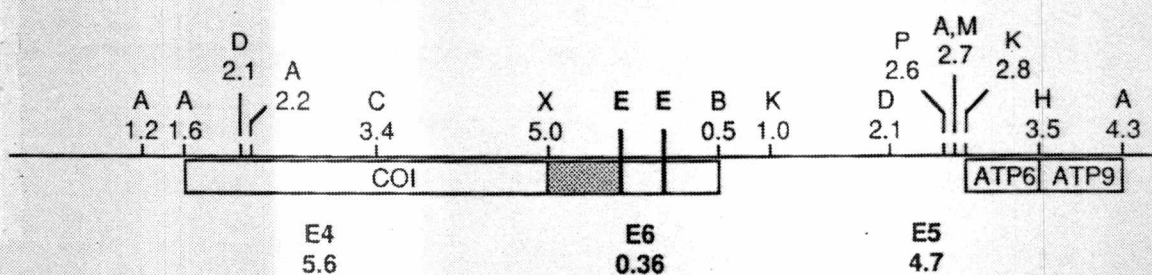
pE4 and pE5 were digested with the indicated restriction endonucleases, electrophoresed in 1% agarose gels and Southern blotted. A- Photograph of ethidium bromide-stained gel. B- Autoradiograph of Southern blot probed with labeled mtDNA from *S. cerevisiae* petite strain DS6 A422. Lane 1, λ HindIII size marker; lane 2, pE4 digested with *Cla*I and *Xba*I; lane 3, pE4 digested with *Ava*I and *Bst*II; lane 4, pE4 digested with *Ava*I; lane 5, pE4 digested with *Eco*RI and *Bst*II; lane 6, pE5 digested with *Bam*HI. Locations of restriction sites are given as the distance in Kb from the left end of the fragment. Symbols for restriction sites are as in Figure 1.



A



B



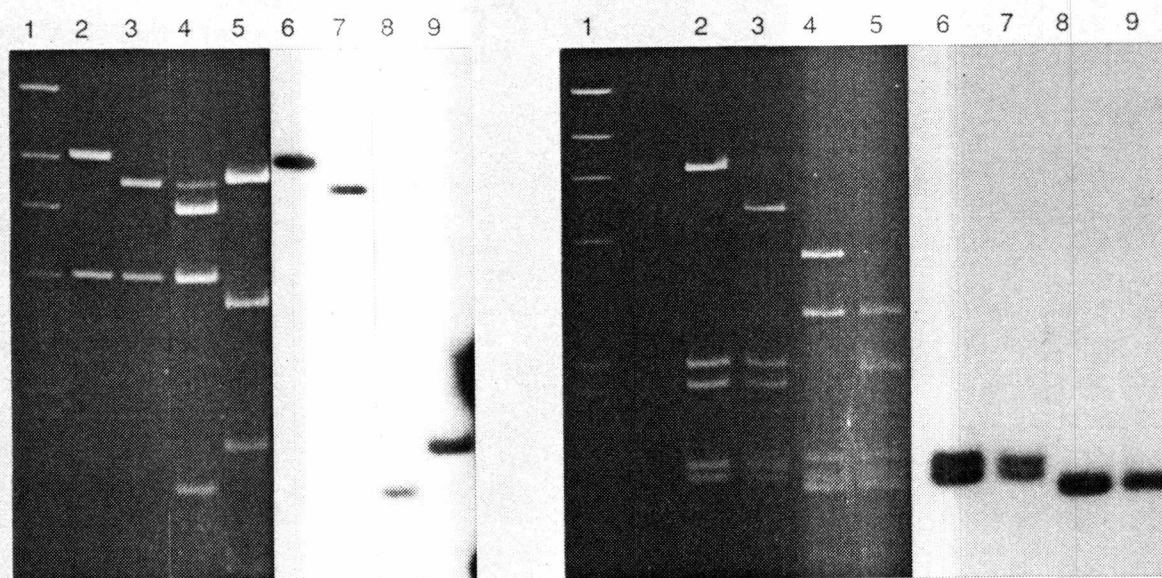
The left-hand end of the sequences homologous to the COI probe was found to be no further left than the *Ava*I site at 1.6Kb on the diagram. This is because hybridization occurs with the 0.6Kb *Ava*I band (between 1.6 and 2.2 on the diagram) but not with the 0.4Kb *Ava*I band between 1.2 and 1.6 (lanes A4 and B4). The gene itself could extend to the left of the indicated boundary since the probe used does not contain the first and second exons of the *S. cerevisiae* wild-type gene (1). However, it should be noted that the polarity of the gene in *C. albicans* is not deducible from these data.

The portion of E5 homologous to the probe was determined by digesting pE5 with *Bam*HI producing two bands: an 8.2Kb band consisting of 4.2Kb of E5 plus 4.0Kb of vector DNA and a 0.9Kb band consisting of 0.5Kb of E5 plus 0.365Kb of vector (lane A6). The probe hybridized only to the smaller fragment, indicating homology does not extend beyond the *Bam*HI site at 0.5Kb on the diagram. The argument described previously concerning the end of the actual gene applies here as well.

When the *C. albicans* mtDNA *Eco*RI fragments were probed with mtDNA from strain DS302 (Cytochrome oxidase subunit II, or COII) hybridization was observed with E2 and E3 (Fig. 3 lanes A1 and A2). This could either mean that the gene is within the inverted duplication and therefore exists in two copies or that the gene straddles the junction of fragments E2 and E3. The latter was found to be the case. In lane A4, pE3 was digested with *Eco*RI and *Kpn*I cutting the fragment into a 6.9Kb and a 1.3Kb fragment (leaving the intact E3 fragment as a partial digestion product). Hybridization was observed only with the 1.3Kb *Kpn*I/*Eco*RI fragment (lane A8).

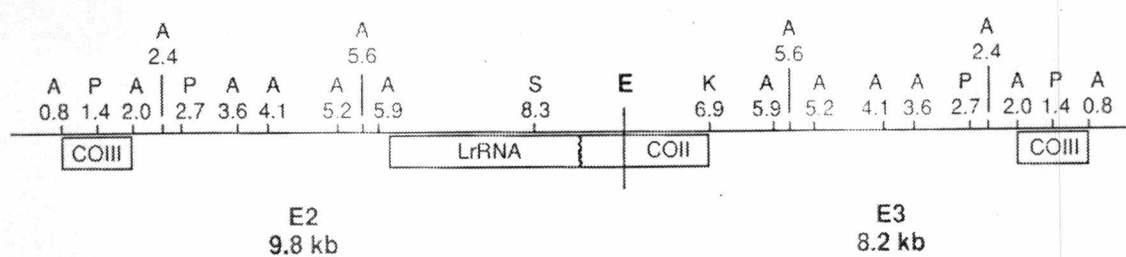
Figure 3. Location of Cytochrome Oxidase subunits II and III.

pE2 and pE3 were digested with the indicated restriction endonucleases, electrophoresed in 1% agarose gels and Southern blotted. A- Photograph of ethidium bromide-stained gel (lanes 1-5) and autoradiograph of Southern blot probed with labeled mtDNA from *S. cerevisiae* petite strain DS302 (COII) (lanes 6-9). Lane 1, λ HindIII size marker; lane 2, pE2 digested with *EcoRI*; lane 3, pE3 digested with *EcoRI*; lane 4, pE3 digested with *EcoRI* and *KpnI* (partial digest); lane 5, pE2 digested with *EcoRI* and *SalI*; lanes 6-9, autoradiograph of lanes 2-5, respectively. B- Photograph of ethidium bromide-stained gel (lanes 1-5) and autoradiograph of Southern blot probed with labeled mtDNA from petite strain DS31 (COIII) (lanes 6-9). Lane 1, λ HindIII size marker; lane 2, pE2 digested with *EcoRI* and *PvuII*; lane 3, pE3 digested with *EcoRI* and *PvuII*; lane 4, pE2 digested with *EcoRI* and *AvaI*; lane 5, pE3 digested with *EcoRI* and *AvaI*; lanes 6-9, autoradiograph of lanes 2-5, respectively. Locations of sites are indicated as distances in Kb from right end of E3 and left end of E2. Some restriction sites omitted for clarity. Symbols for restriction sites are as in Figure 1.



A

B



pE2 was digested with *EcoRI* and *SaII* (lane A5) and hybridization was limited to the 1.5Kb *SaII/EcoRI* fragment (lane A9). This confirms that the COII gene straddles the *EcoRI* site at the junction of fragments E2 and E3. It should be noted that the cytochrome oxidase II gene cannot extend all the way to the *SaII* site in E2 since it has already been shown that the large ribosomal RNA gene extends to the right of this site (see Part 2, pg. 20). This uncertainty regarding the relative locations of the ends of the two genes is represented by the jagged line separating the genes in the diagram at the bottom of Figure 3.

When fragments E1 - E6 were probed with mtDNA from strain DS31 (Cytochrome oxidase subunit III or COIII) hybridization was again observed with fragments E2 and E3 (data not shown). When pE2 and pE3 were digested with *PvuII* (lanes B2 and B3 respectively) hybridization was observed with two fragments of 1.4 and 1.3Kb (lanes B6 and B7). These fragments are entirely within the inverted duplication and thus exist in both E2 and E3. The COIII gene was further localized to a 1.2Kb *AvaI* fragment (lanes B8 and B9).

Location of the gene encoding Cytochrome b

Initially the probe used to locate the cytochrome b gene was mtDNA from petite strain DS400 A12 which carries the entire cytochrome b gene of *S. cerevisiae*. Hybridization signals were observed from fragments E1, E2 and E4 (data not shown). Hybridization with these three fragments indicated a widely dispersed gene interrupted by the small ribosomal RNA gene (see

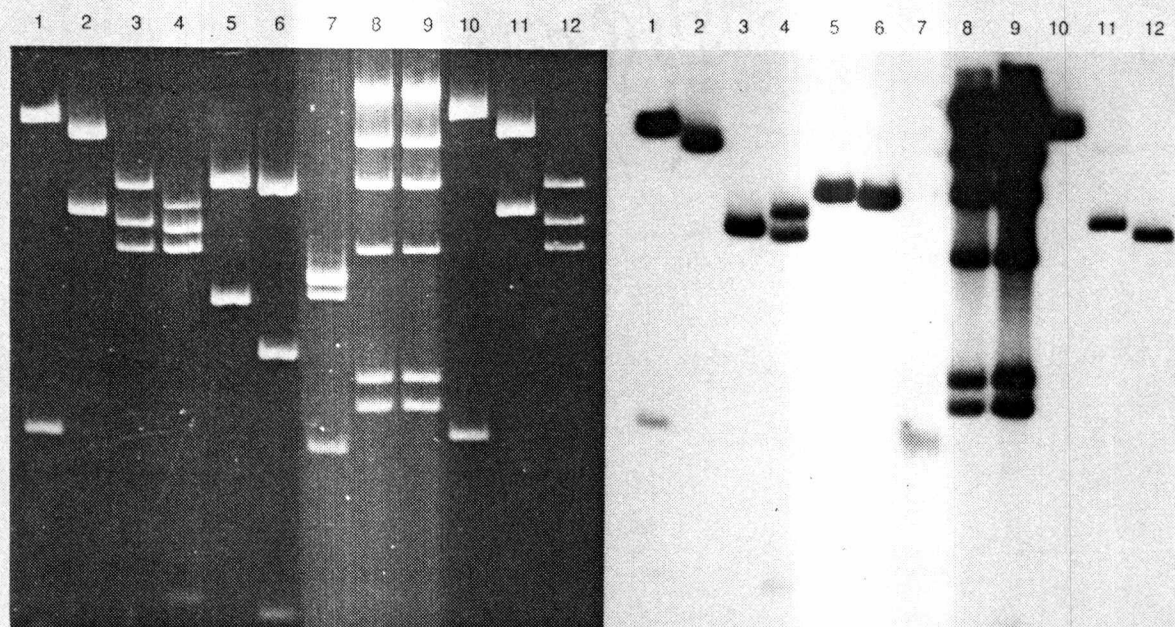
Part 2). This would be a very unusual situation. If the hybridization with E2 were disregarded (which is reasonable since the signals were weak and might therefore be attributed to random cross-hybridization) then the E1/E4 hybridization could be explained by the gene straddling the *EcoRI* site at the E1/E4 junction. However, further examination showed that the probe was hybridizing with the end of E4 distal to the E1/E4 junction (data not shown). This is the same end of E4 that was earlier postulated to contain intron sequences.

To resolve this curious situation two subgenic probes carrying different portions of the cytochrome b gene were used. mtDNA from petite strain DS400 M4 contains the sequences for exon 1, intron 1, exon 2, and part of intron 2 while strain DS400 M4 has sequences for exon 3 as well as a large amount of downstream A-T rich sequences (16). When both probes were used against fragments E1, E2 and E4 only the exons 1 and 2 probe hybridized to all three fragments while the exon 3 probe only hybridized to fragment E1. Therefore it is likely that cross-hybridization to fragments E2 and E4 is caused by a combination of some sequence similarity (perhaps between intron regions) and the low stringency hybridization conditions used. It remained to precisely locate the gene on E1 and to explain more thoroughly why the exons 1 and 2 probe hybridized to fragments E4 and E2.

pE1 was digested with *HindIII* and probed with exons 1 and 2 (Fig. 4, lane B1) and exon 3 (lane B10). The large band consists of E1 DNA to the right of the *HindIII* site at 1.7Kb plus vector DNA and the smaller band represents the remainder of E1. The exons 1 and 2

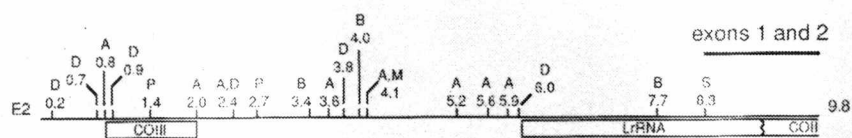
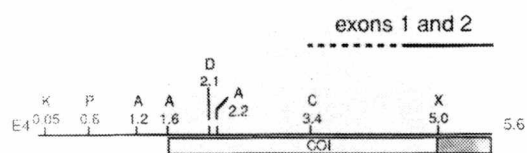
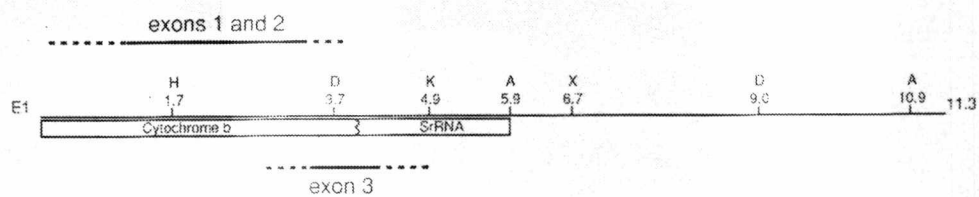
Figure 4. Location of the gene encoding Cytochrome b.

pE1, pE2 and pE4 were digested with the indicated restriction endonucleases, electrophoresed in 1% agarose gels and Southern blotted. A- Photograph of ethidium bromide-stained gel. Lane 1, pE1 digested with *Hind*III; lane 2, pE1 digested with *Bst*EII; lane 3, pE1 digested with *Eco*RI and *Kpn*I; lane 4, pE4 digested with *Eco*RI and *Xba*I; lane 5, pE4 digested with *Cla*I; lane 6, pE4 digested with *Ava*I; lane 7, pE2 digested with *Sal*I and *Bam*HI; lanes 8 and 9, λ *Hind*III size marker; lanes 10-12, same as lanes 1-3. Autoradiograph of Southern blot probed with labeled mtDNA from *S. cerevisiae* petite strain DS400 M4 (cytochrome b exons 1 and 2)(lanes 1-7) and from strain DS400 M11 (cytochrome b exon 3)(lanes 10-12). Diagram at bottom of figure shows interpretation of results. Heavy lines indicate probable extent of sequences hybridizing to the probe as deduced from the intensity of the hybridization signals while dotted portions indicate the maximum possible extent of those sequences. Symbols for restriction sites are as in Figure 1.



A

B



probe hybridizes to both fragments (lane B1) indicating that homology to the probe straddles the *HindIII* site. The greater intensity of the signal from the upper band probably results from more sequences homologous to the probe lying to the right of the *HindIII* site than to the left. Only the larger fragment hybridizes with the exon 3 probe (lane B10); therefore there is no exon 3 homology to the left of the *HindIII* site. pE1 was digested with *BstEII* (lanes A2 and A11) producing a 5.3Kb internal fragment and a 10.4Kb E1/pBr322 junction fragment. Hybridization to the smaller band indicates probe homology extending to the right of the *BstEII* site at 3.7Kb from the E1/E4 junction. The exons 1 and 2 probe hybridizes only to the larger fragment (lane B2) while the exon 3 probe hybridizes predominantly with the smaller fragment (lane B11). Thus the polarity of the cytochrome b gene can be deduced as follows: the 5' end of the gene is somewhere between the *EcoRI* site at the E1/E4 junction and the *HindIII* site at 1.7Kb. The 3' end is between the *BstEII* site at 3.7Kb and the *KpnI* site at 4.9Kb since the SrRNA gene has already been shown to extend to the left of that *KpnI* site (see Part 2, pg. 23).

Several digests of pE4 were probed with the exons 1 and 2 probe in order to determine where the cross-hybridization was occurring. In lane A4 of Figure 4, pE4 was digested with *EcoRI* and *XbaI* (the *XbaI* digest is incomplete) and hybridization was observed with both the 5.0Kb fragment and the 0.6Kb putative COI intron fragment (lane B4). Further examination using *ClaI* (lanes A5 and B5) shows that the homology is confined to the right of the *ClaI* site in E4.

pE2 was digested with *Sal*I and *Bam*HI (lane A7) and the exons 1 and 2 probe hybridized only with the 1.8Kb *Sal*I/*Bam*HI E2/pBr322 junction fragment (lane B7). The cross-hybridization in this case could be either with LrRNA or COII sequences.

Identification of *C. albicans* mitochondrial RNA transcripts

As a prelude to future experiments dealing with the expression of *C. albicans* mitochondrial genes we undertook the isolation and initial analysis of the mitochondrial mRNAs. When Northern blots were probed with the various single-gene petite-derived probes, no transcripts were visualized. This was due mainly to hybridizations and subsequent washes being done at such low stringency that background hybridization reduced the signal to noise ratio below acceptable levels. As an alternative approach, *C. albicans* mtDNA restriction fragments specific for each previously mapped gene were isolated from agarose gels by electroelution. These fragments described in METHODS were labeled and used as gene-specific probes. A Northern blot of *C. albicans* mtRNA was cut into strips and each strip probed with one of these gene-specific probes, Figure 5. Lanes 1 and 2 were probed with the small and large ribosomal RNA probes, respectively. The estimated size of the SrRNA transcript is 1.35Kb and that of the LrRNA is 2.37Kb. The cytochrome b gene (lane 3) appears to have 4 major transcripts ranging in size from about 500 bp to about 1700. The smaller two transcripts are considerably smaller than the mature cytochrome b transcripts from the more thoroughly characterized yeasts *S. cerevisiae* (16) and

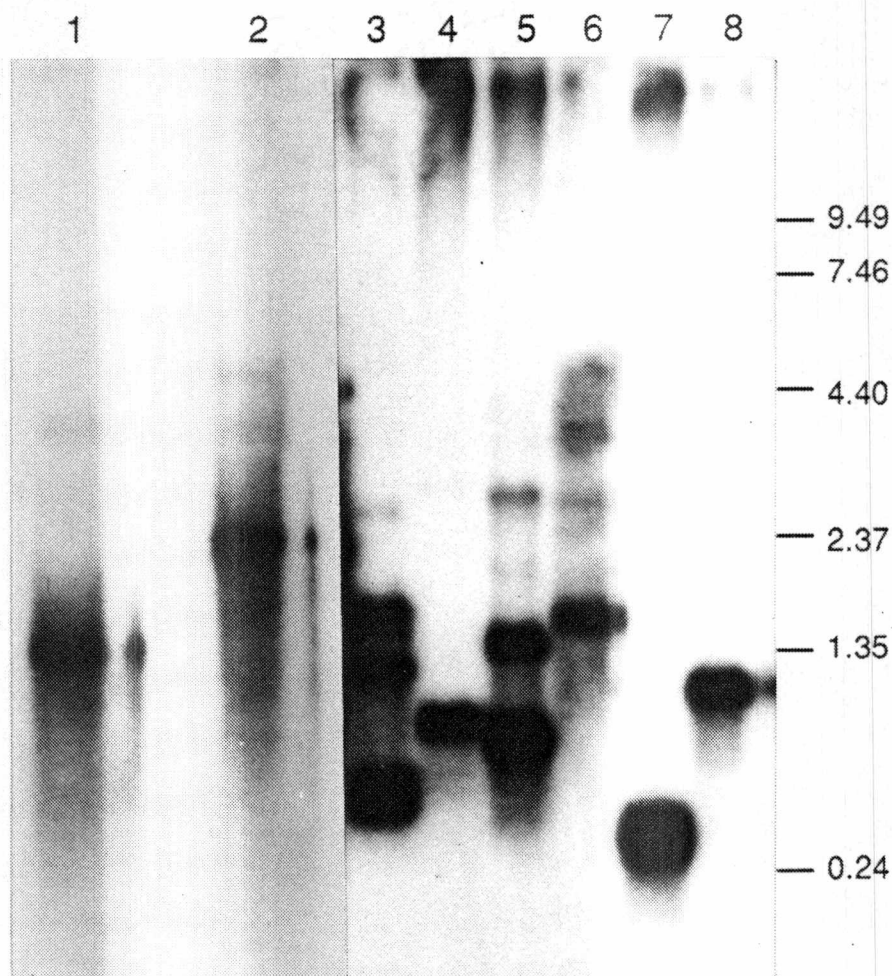


Figure 5. Expression of *Candida albicans* mitochondrial genes.

Autoradiograph of Northern blot of *C. albicans* mitochondrial RNA probed with *C. albicans*-derived gene-specific DNA probes. Probes for each lane are as follows: Lane 1, SrRNA; lane 2, LrRNA; lane 3, Cyt. b; lane 4, COIII; lane 5, COII; lane 6, COI; lane 7, ATPase subunit 9; lane 8, ATPase subunit 6. Gel lane containing RNA size markers was removed before blotting and stained with ethidium bromide and photographed. Sizes given are in Kb. Lanes 1 and 2 are from a separate gel run under identical conditions.

Torulopsis glabrata (6). However the larger two transcripts of 1700 bp and 1100 bp are reasonably near the common size of about 1300 bp. The smaller transcripts could arise in one of several ways. First, it should be noted that the probe in this case is a 1.7Kb DNA fragment derived from *EcoRI* fragment E1 digested with *HindIII*. However, the 5' end of the cytochrome b gene does not necessarily extend all the way to the *EcoRI* site (see Fig. 4). Therefore this probe may overlap the coding sequence of another gene (as yet unidentified) adjacent to the cytochrome b gene. It is possible that tRNA genes, ribosomal protein genes (such as *var 1*), or NADH dehydrogenase genes are present in this area. Secondly, the small transcripts could be RNA processing products such as spliced out introns or the exon/intron message for an RNA splicing enzyme (16).

The cytochrome oxidase subunit 3 (COIII) probe hybridized to a single transcript of about 900 bp (lane 4) while the cytochrome oxidase subunit 2 (COII) probe reveals two transcripts of about 1400 bp and 800 bp and a minor transcript of about 2900 bp (lane 5). There is no precedent for an intron in the COII gene; therefore without further evidence such as sequence information or S1 nuclease analysis the possibility of the probe having dual specificity for COII and some other gene's transcript must be considered here as it was for cytochrome b.

The cytochrome oxidase subunit 1 probe (COI) hybridizes strongly to a single transcript of about 1700 bp and more weakly to several transcripts increasing in size from about 2800 bp to about 5000 bp (lane 6). The size of the primary transcript is consistent with its observed size in *T. glabrata* (6) and in *S. cerevisiae* (1). The

larger, less abundant transcripts are probably processing intermediates given that we already have indirect evidence of at least one intron (see Fig. 2) and the fact that this gene usually contains at least one intron (9,2,15,7,14).

The ATPase subunit 9 and 6 probes hybridized to one transcript each (lanes 7 and 8 respectively) of about 400 bp for subunit 9 and about 1100 bp for subunit 6. These sizes correspond closely to sizes of the same transcripts in *T. glabrata* (6).

DISCUSSION

Figure 6 summarizes the mapping data presented in this paper. The organization of genes on the *C. albicans* mtDNA represents yet another example of the variability of fungal mitochondrial genomes as shown in Figure 7. As we previously reported, the ribosomal RNA genes are not located in the inverted duplication (19) which is unusual for an organelle genome having such a structure. Another unusual feature is the presence of the gene encoding cytochrome oxidase subunit III within the inverted duplication. This also has not been observed previously. There are also many features that the *C. albicans* mitochondrial genome has in common with other mitochondrial genomes. Clark-Walker and Sriprakash (4) noted that the ATPase subunit 6 and 9 genes were juxtaposed in *Torulopsis glabrata* and in *Saccharomyces exiguus* and speculated about possible reasons for this. We have shown that this juxtaposition also exists in *C. albicans* mtDNA. Investigation of the expression of these genes showed that, just as in *T. glabrata*, each gene has a unique transcript (Figure 5).

Some closely related species in the *Dekkera/Brettanomyces* genera have been found to have identical mitochondrial gene organization (10). Other than this example very few fungal mitochondrial genomes have been found with similar gene orders. When we compared the *C. albicans* mitochondrial genome to the handful of other mapped genomes we found it to be very similar to the genomes of *Saccharomyces exiguus* and *Torulopsis glabrata* (3,5).

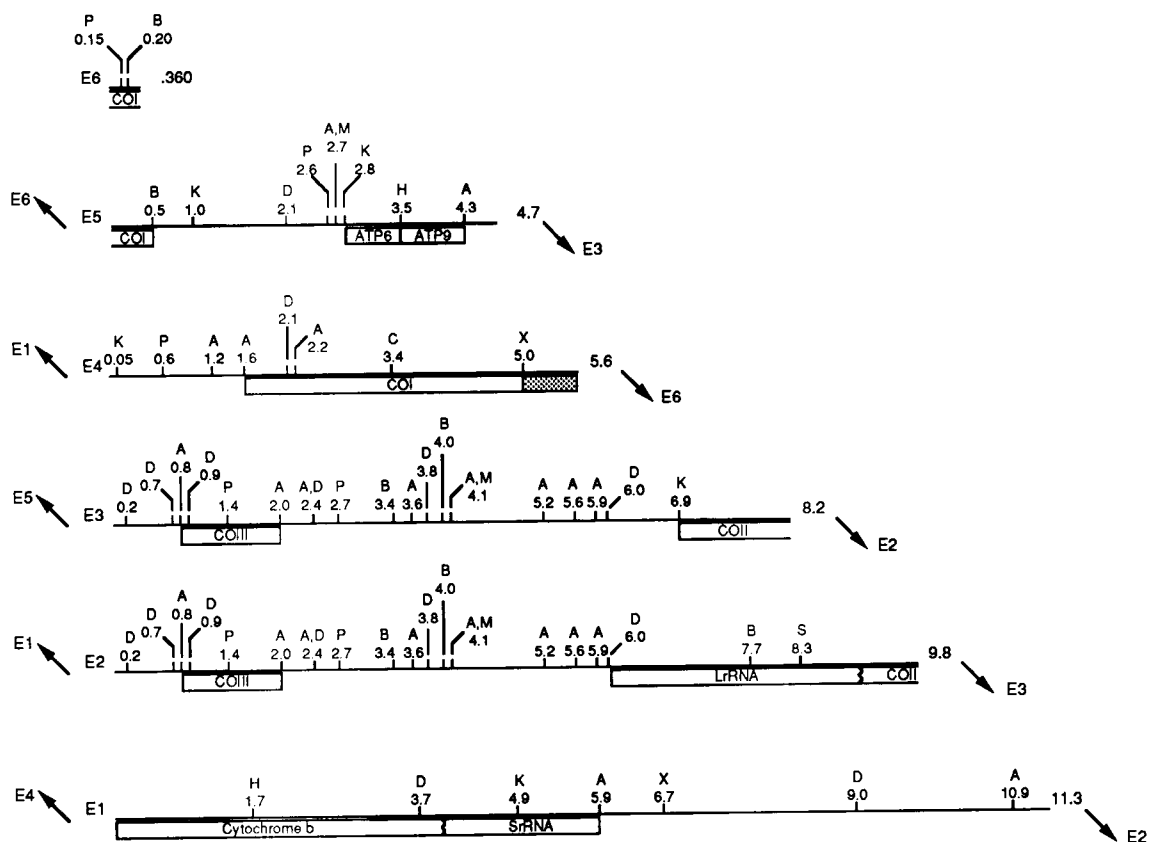


Figure 6. Mitochondrial genes placed on the restriction map of *Candida albicans* cloned *Eco*RI fragments.

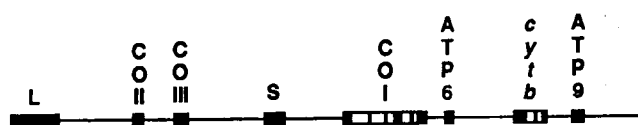
Arrows at the termini of each fragment are included to indicate the adjacent fragment in the whole molecule. Symbols for restriction sites are as in Figure 1. Wavy lines indicate that two genes terminate on the same restriction fragment but the exact positions of the termini are unknown.

Figure 7. Comparison of mitochondrial DNA size and gene order among seven fungi.

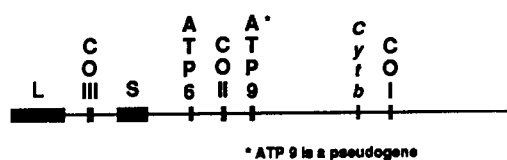
Mitochondrial maps were linearized arbitrarily at the large rRNA gene and oriented left to right by that direction giving the shortest distance to the small rRNA gene. The size of each molecule in Kb is given under the name of each fungus. References for individual maps are as follows: *S. cerevisiae* (9), *N. crassa*, *K. lactis*, *K. africana*, *T. glabrata* (20), *S. exiguus* (3).

Saccharomyces cerevisiae

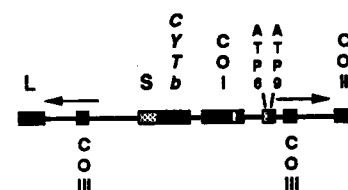
75

*Neurospora crassa*

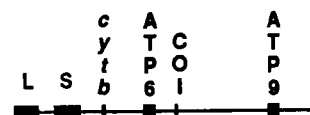
60

*Candida albicans*

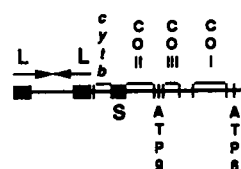
40

*Kluyveromyces lactis*

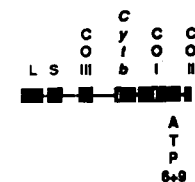
37

*Kloeckera africana*

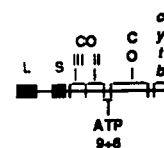
27

*Saccharomyces exiguus*

24

*Torulopsis glabrata*

18



As shown in Figure 8 there are only two differences between the *C. albicans* order and the *S. exiguus* order. The COIII gene is carried in the inverted duplication and therefore one of its copies has no corresponding element in the *S. exiguus* genome. The other difference involves the other copy of the COIII gene and the small ribosomal RNA gene. The order of these two genes in *C. albicans* is reversed with respect to the same genes in *S. exiguus*. This striking similarity could be the result of random shuffling. However these observations do correlate well with Clark-Walker's hypothesis regarding the evolution of mitochondrial genomes (3). He speculates that the ancestral mitochondrial genome was small with closely spaced genes and that evolution has produced larger and larger genomes with concurrent rearrangement of genes and the introduction of introns and non-coding spacer DNA. This model assumes that because of its size the 18Kb genome of *T. glabrata* is closer to the ancestral form than larger genomes. Only one change is necessary to get the order seen in the 27Kb *S. exiguus* genome and one more change (disregarding the inverted duplication) to get the order seen in the 40Kb *C. albicans* mitochondrial genome (Figure 8).

Examination of the expression of the *C. albicans* mitochondrial genes showed that each gene is probably transcribed separately since no two transcripts or precursor transcripts were found of the same size. No cotranscription has been observed (6). The estimated size of each transcript is reasonably close to that of the corresponding transcripts of *T. glabrata* (6).

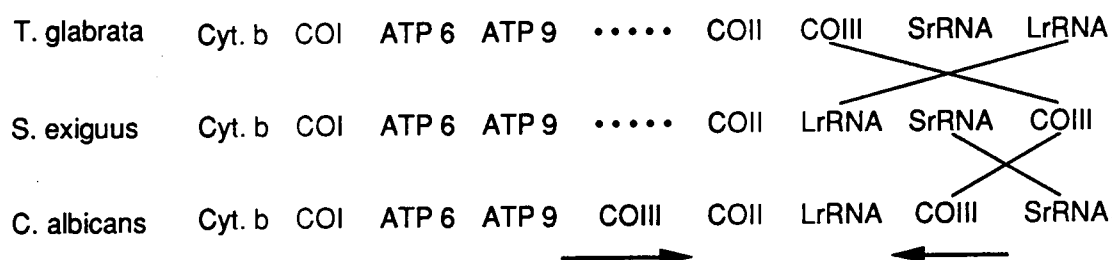


Figure 8. Comparison of mitochondrial gene orders.

Circular gene maps for each fungus were linearized arbitrarily at the cytochrome b gene. Compensation for the inverted duplicated region of *C. albicans* is indicated by ellipses in the *T. glabrata* and *S. exiguus* maps. Diagonal lines indicate the changes necessary to generate one gene order from the previous one.

Three genes were found possibly to contain introns since higher molecular weight transcripts producing bands of lower intensity were observed in the Northern blots. Of these three genes in other fungi, the COI gene has always been found to contain introns and the cyt. b gene commonly contains introns (9,2,15,7,14). However, introns have not previously been detected in the COII gene. As mentioned earlier the DNA fragment used as a probe in each case could conceivably overlap the sequence of an as yet unidentified gene and thereby possess dual specificity. It will be necessary to sequence and/or do S1 nuclease analysis of these genes in order to confidently demonstrate their possession of introns.

If these genes do contain introns it is possible that the mRNA maturation system is similar to that seen in *S. cerevisiae*. In that case, intron 1 of cytochrome b is 70% similar to intron 4 of cytochrome oxidase subunit 1 (1). We have found that the DNA sequence of *C. albicans* fragment E6 (J. A. Shaw, unpublished, and R. Poulter, personal communication) contains a sequence similar to the 3' end of exon 4 of COI. However, we do not observe similarity between the remainder of E6 and either cytochrome b intron 1 or intron 4 of COI. Yet, the E6-proximal end of fragment E4 does not hybridize to the COI probe but it does hybridize with the cytochrome b exons 1 and 2 probe. This could be the result of some sort of transposition of *C. albicans* intron sequences from the cytochrome b gene to the COI gene. This sequence then remained similar enough to the *S. cerevisiae* intron to be detected by low stringency hybridization but did not retain that level of similarity to the COI intron 4 sequence of *S. cerevisiae*.

LITERATURE CITED

1. Bonitz, S. G., G. Coruzzi, B. E. Thalenfeld, A. Tzagoloff, and G. Macino. 1980. Assembly of the mitochondrial membrane system. Structure and nucleotide sequence of the gene coding for subunit 1 of yeast cytochrome oxidase. *J. Biol. Chem.* 255: 11927-11941.
2. Brown, T. A., R. B. Waring, C. Scazzocchio, and R. W. Davies. 1985. The *Aspergillus nidulans* mitochondrial genome. *Curr. Genet.* 9: 113-117.
3. Clark-Walker, G. D., C. R. McArthur and K. S. Sriprakash. 1983. Order and orientation of genic sequences in circular mitochondrial DNAs from *Saccharomyces exiguus*: Implications for evolution of yeast mtDNAs. *J. Mol. Evol.* 19: 333-341.
4. Clark-Walker, G. D. and K. S. Sriprakash. 1981. Sequence rearrangements between mitochondrial DNAs of *Torulopsis glabrata* and *Kloeckera africana* identified by hybridization with six polypeptide encoding regions from *Saccharomyces cerevisiae* mitochondrial DNA. *J. Mol. Biol.* 151: 367-387.
5. Clark-Walker, G. D. and K. S. Sriprakash. 1983a. Analysis of a five gene cluster and unique orientation of large genic sequences in *Torulopsis glabrata* mitochondrial DNA. *J. Mol. Evol.* 19: 342-345.
6. Clark-Walker, G. D. and K. S. Sriprakash. 1983b. Map location of transcripts from *Torulopsis glabrata* mitochondrial DNA. *EMBO J.* 2: 1465-1472.
7. Collins, R. A. and A. M. Lambowitz. 1983. Structural variations and optional introns in the mitochondrial DNAs of *Neurospora crassa* strains isolated from nature. *Plasmid* 9: 53-70.
8. Coruzzi, G., S. G. Bonitz, B. E. Thalenfeld, and A. Tzagoloff. 1981. Assembly of the mitochondrial membrane system. Analysis of the nucleotide sequence and transcripts in the *oxi1* region of yeast mitochondrial DNA. *J. Biol. Chem.* 256: 12780-12787.

9. Dujon, B. 1981. Mitochondrial genetics and function. in *The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance*. (J. N. Strathern, E. W. Jones, J. R. Broach, eds.; New York: Cold Spring Harbor Laboratory) pp. 505-635.
10. Hoeben, P., and G. D. Clark-Walker. (1986). An approach to yeast classification by mapping mitochondrial DNA from *Dekkera/Brettanomyces* and *Eeniella* genera. *Curr. Genet.* 10: 371-379.
11. Macino, G. and A. Tzagoloff. 1979. Assembly of the mitochondrial membrane system. The DNA sequence of a mitochondrial ATPase gene in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 254: 4617-4623.
12. Macino, G. and A. Tzagoloff. 1980. Assembly of the mitochondrial membrane system: Sequence analysis of a yeast mitochondrial ATPase gene containing the *oli-2* and *oli-4* Loci. *Cell* 20: 507-517.
13. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
14. Merlos-Lange, A. M., F. Kanbay, M. Zimmer, and K. Wolf. 1987. DNA splicing of mitochondrial group I and II introns in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* 206: 273-278.
15. Nelson, M. A. and G. Macino. 1985. Gene organization and expression in *Neurospora crassa* mitochondria. in *Achievements and Perspectives of Mitochondrial Research*. Vol. II: *Biogenesis*. (E. Quagliariello, E. C. Slater, F. Palmieri, C. Saccone, A. M. Kroon, eds.; New York: Elsevier) pp. 293-304.
16. Nobrega, F. G. and A. Tzagoloff. 1980. Assembly of the mitochondrial membrane system. DNA sequence and organization of the cytochrome b gene in *Saccharomyces cerevisiae* D273-10B. *J. Biol. Chem.* 255: 9828-9837.

17. Thalenfeld, B., and A. Tzagoloff. 1980. Assembly of the mitochondrial membrane system. Sequence of the *Oxi2* gene of yeast mitochondrial DNA. *J. Biol. Chem.* 255: 6173-6180.
18. Torres Bauza, L. J., and W. S. Riggsby. 1980. Protoplasts from yeast and mycelial forms of *Candida albicans*. *J. Gen. Microbiol.* 119: 341-349.
19. Shaw, J. A., W. B. Troutman, B. A. Lasker, M. M. Mason and W. S. Riggsby. The inverted duplication in the mitochondrial DNA of *Candida albicans*. Submitted to *J. Bacteriol.*
20. Wallace, D. C. 1982. Structure and evolution of organelle genomes. *Microbiol. Rev.* 46: 208-240.
21. Wills, J. W., W. B. Troutman, and W.S. Riggsby. 1985. Circular mitochondrial genome of *Candida albicans* contains a large inverted duplication. *J. Bacteriol.* 164: 7-13.

PART 4. CONCLUSION

RESULTS AND PROSPECTIVES

Summary of results

The 40Kb mtDNA of *Candida albicans* contains sequences that hybridize to all eight of the *Saccharomyces cerevisiae* mitochondrial genes tested. Figure 1 shows a circular representation of the mapping data. Unusual structural features of the *C. albicans* mtDNA include the presence of an inverted duplication and the fact that both rRNA genes are not coded for in the duplicated region. While common in chloroplast DNA's, inverted duplications are uncommon in mtDNA's with only two other fungal mtDNA's possessing them out of the hundreds of mtDNA's examined. When organelle genomes do contain inverted duplications all or part of the rRNA genes have been found within these regions; thus *C. albicans* is the only known example in which this is the case.

C. albicans mtDNA also possesses structural features similar to other mtDNA's. The ATPase subunit 6 and 9 genes are juxtaposed just as they are in *Torulopsis glabrata* and *Saccharomyces exiguus* (3,4). Additionally the relative order of genes in *C. albicans* is very similar to the gene order in *S. exiguus*. These observations are in perfect agreement with Clark-Walker's hypothesis regarding the evolution of mitochondrial genomes (3). The putative, small ancestral mitochondrial genome represented by that of *T. glabrata* is one change away from the larger *S. exiguus* genome which in turn requires one more change (plus the acquisition of an inverted duplication) to get the order seen in the even larger *C. albicans*

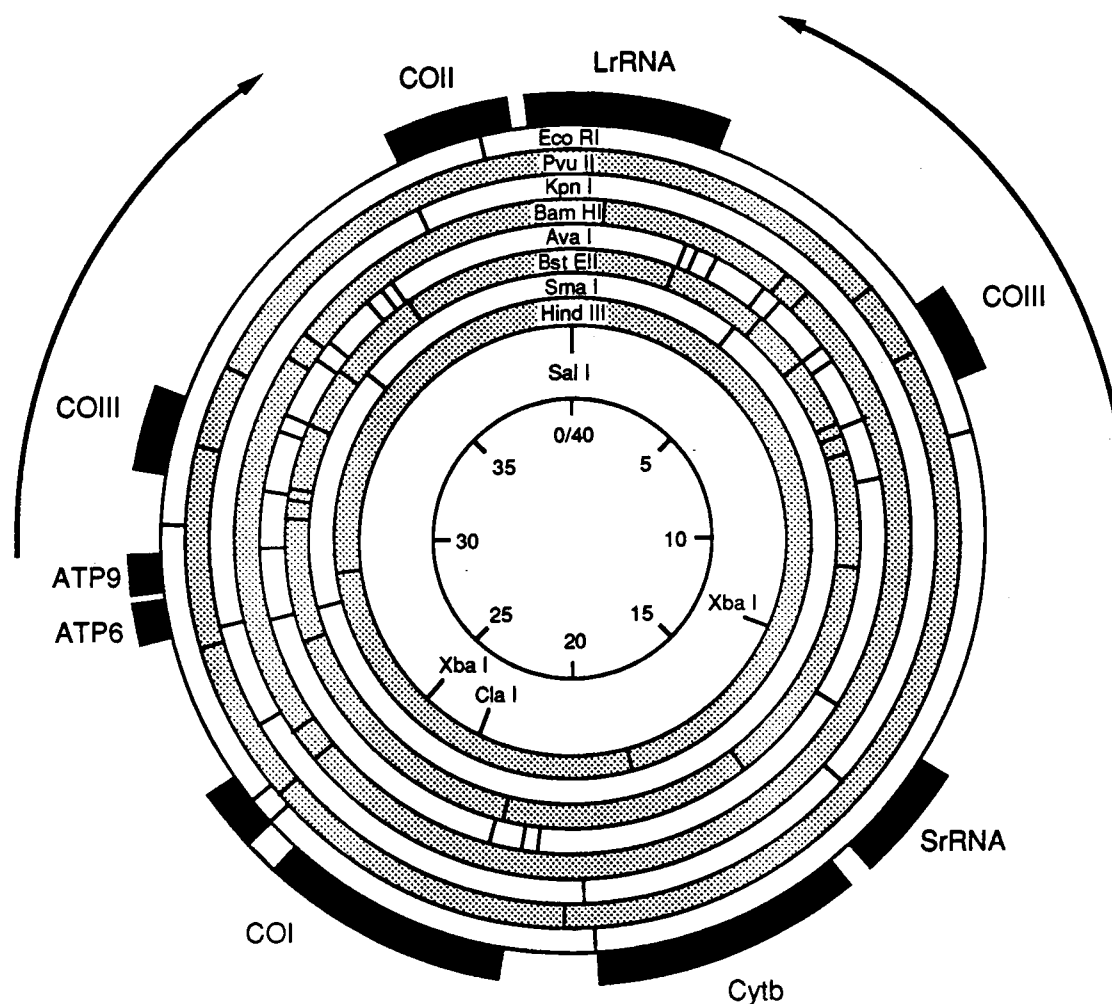


Figure 1. Circular map of the mitochondrial DNA of *Candida albicans*.

The scale on the innermost ring is in Kb and starts arbitrarily at the single *Sal*I site. Locations of the genes are indicated by black boxes on the outermost ring. Heavy black arrows designate the location of the inverted duplication.

mitochondrial genome. This theory generates an interesting question concerning a fundamental difference between fungal mtDNA's and metazoan mtDNA's. In the course of evolution, did fungal mtDNA's lose control of size and gene arrangement or did metazoan mtDNA's gain that control? In view of the ancient divergence of the various fungal groups, which in itself ensures great genomic diversity, and the relatively few fungal genomes that have been studied, the answers to these questions must for the present be entirely speculative. In any case, the elucidation of such evolutionarily diverse taxa will be far better pursued by studying highly conserved sequences such as rRNA genes than by comparisons of mitochondrial gene order.

There appears to be nothing unusual about the expression of the *C. albicans* mitochondrial genes. Transcripts have been identified for each gene and the sizes of these transcripts compare favorably with the homologous transcripts of other fungi. Three genes appear to have introns since bands corresponding to processing intermediates and/or products of splicing reactions have been observed in Northern blots.

Directions of future research

There are a few remaining mitochondrial genes for which heterologous probes are available or can be derived. These include the gene for the ribosomal protein *var1* in *S. cerevisiae* (1) and a set of NADH dehydrogenase genes that has been found in the mtDNA's of

animals (2) and some of the filamentous fungi (6). These NADH dehydrogenase genes are not found in *S. cerevisiae* or the other yeasts so it will be necessary to determine their presence or absence in *C. albicans* mtDNA. Of course all the *C. albicans* mitochondrial genes should be sequenced for comparative purposes and the intergenic spaces should be sequenced to search for unidentified open reading frames and tRNA genes.

With the mitochondrial mRNA's identified it will be possible to monitor the expression of mitochondrial genes under different conditions. Such conditions would include the presence of antifungal drugs, inhibitors of various steps in respiration or different carbon sources. It will also be possible to determine if there is a mitochondrial role in the yeast to mycelial conversion or in the recently documented phenomenon of phenotypic switching in which *C. albicans* colonies undergo a reversible change in morphology (7).

The mechanisms of mRNA maturation so thoroughly examined in *S. cerevisiae* may be present in *C. albicans* in an enhanced or less developed form. Therefore the introns should be sequenced for comparison with the *S. cerevisiae* introns. Furthermore, with the recently developed method of mitochondrial transformation by projectile bombardment (5) it will be possible to determine whether *C. albicans* intron-encoded proteins can complement certain splicing-deficient mutations of *S. cerevisiae* mtDNA.

LITERATURE CITED

1. Butow, R., P. Perlman, and L. Grossman. 1985. The unusual *var1* gene of yeast mitochondrial DNA. *Science* 228: 1496-1501.
2. Chomyn, A., P. Mariottini, M.W.J. Cleeter, C.I. Ragan, A. Matsuno-Yagi, Y. Hatefi, R. F. Doolittle, and G. Attardi. 1985. Six unidentified reading frames of human mitochondrial DNA encode components of the respiratory-chain NADH dehydrogenase. *Nature* 314: 592-597.
3. Clark-Walker, G. D., C. R. McArthur and K. S. Sriprakash. 1983. Order and orientation of genic sequences in circular mitochondrial DNAs from *Saccharomyces exiguus*: Implications for evolution of yeast mtDNAs. *J. Mol. Evol.* 19: 333-341.
4. Clark-Walker, G. D. and K. S. Sriprakash. 1983. Analysis of a five gene cluster and unique orientation of large genic sequences in *Torulopsis glabrata* mitochondrial DNA. *J. Mol. Evol.* 19: 342-345.
5. Johnston, S. A., P. Q. Anziano, K. Shark, J. C. Sanford, and R. A. Butow. 1988. Mitochondrial transformation in yeast by bombardment with microprojectiles. *Science* 240: 1538-1541.
6. Nelson, M. A. and G. Macino. 1987. Structure and expression of the overlapping ND4L and ND5 genes of *Neurospora crassa* mitochondria. *Mol. Gen. Genet.* 206: 307-317.
7. Slutsky, B., J. Buffo, and D. R. Soll. 1985. High-frequency switching of colony morphology in *Candida albicans*. *Science* 230: 666-669.

VITA

The author was born December 5, 1960 in Atlanta, Georgia. His interest in science was sparked early by frequent visits to the Fernbank Science Center across the street from his elementary school. There at the age of ten he saw his first mitochondrion at the electron microscope facility. At Druid Hills High School his biology teacher gave him the opportunity to compete for a summer job at the nearby Centers for Disease Control. Winning this honor marked the start of his career in microbiology. In 1980, he matriculated at the University of Tennessee, Knoxville where he majored in microbiology and graduated with highest honors in 1983. Another summer job competition resulted in a technical position in the wastewater treatment laboratory of Tennessee Eastman Company in Kingsport. He was married to Mary Weinberg of Atlanta that summer. He remained at UTK and entered graduate school in the Microbiology Department. He has accepted a post-doctoral position at the National Institutes of Health in Bethesda, Maryland.