Candida albicans mitochondrial dynamics during a yeast to hyphal transition
by Scott D Kobayashi

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
Candida albicans mitochondria (mt) undergo morphological changes during cell cycle events, however, the genetic basis for this change is not well understood. Electrophoresis of C. albicans total cellular DNA yields several intense ethidium bromide-stained moderately sized (>2.5 kilobase pairs) MspI (C/CGG) fragments. These apparent repeat units are shown to be mt in origin. MspI digests of mtDNA were cloned into the Clal site of pBluescript II. Four different mt-specific clones were chosen as probes to determine relative mt genome copy number in C. albicans wild-type and mutant strains grown in a serum-containing medium. The cloned fragments were radiolabeled and used to probe dot blots of total cellular DNA digests. Hybridization dot intensities were compared against similarly labeled C. albicans single-copy actin DNA to obtain an estimate of mt genome copy number. Stationary phase yeast cells contained approximately 3.4 mt genomes, as compared to 4.8 in 3 hr. germ tubes. These results indicate that mt genome copy number increases over time during a yeast to hyphal morphogenesis. Hyphae-deficient mutants also yielded a higher number of mt genomes than wild-type strains. In addition to mt genome copy number changes, we detected changes in specific activities of both succinate dehydrogenase and cytochrome oxidase. Comparison studies using cellular homogenates and crude mt fractions indicated that individual mt undergo changes in enzyme activity prior to mt division and growth. These findings imply that mt processes are important in the yeast to hyphal transition.
CANDIDA ALBICANS MITOCHONDRIAL DYNAMICS DURING A YEAST TO HYPHAL TRANSITION

by

Scott D. Kobayashi

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology

MONTANA STATE UNIVERSITY-BOZEMAN
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APPROVAL

of a thesis submitted by

Scott D. Kobayashi

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Jim E. Cutler

Approved for the Department of Microbiology

Seth Pincus

Approved for the College of Graduate Studies

Joseph J. Fedock
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Date 9/3/98
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ABSTRACT

*Candida albicans* mitochondria (mt) undergo morphological changes during cell cycle events, however, the genetic basis for this change is not well understood. Electrophoresis of *C. albicans* total cellular DNA yields several intense ethidium bromide-stained moderately sized (>2.5 kilobase pairs) *MspI* (C/CGG) fragments. These apparent repeat units are shown to be mt in origin. *MspI* digests of mtDNA were cloned into the *ClaI* site of pBluescript II. Four different mt-specific clones were chosen as probes to determine relative mt genome copy number in *C. albicans* wild-type and mutant strains grown in a serum-containing medium. The cloned fragments were radiolabeled and used to probe dot blots of total cellular DNA digests. Hybridization dot intensities were compared against similarly labeled *C. albicans* single-copy actin DNA to obtain an estimate of mt genome copy number. Stationary phase yeast cells contained approximately 3.4 mt genomes, as compared to 4.8 in 3 hr germ tubes. These results indicate that mt genome copy number increases over time during a yeast to hyphal morphogenesis. Hyphae-deficient mutants also yielded a higher number of mt genomes than wild-type strains. In addition to mt genome copy number changes, we detected changes in specific activities of both succinate dehydrogenase and cytochrome oxidase. Comparison studies using cellular homogenates and crude mt fractions indicated that individual mt undergo changes in enzyme activity prior to mt division and growth. These findings imply that mt processes are important in the yeast to hyphal transition.
Chapter 1

INTRODUCTION

Increasing numbers of immunocompromised individuals have resulted in a rising incidence of disease attributed to opportunistic pathogens, including those caused by fungal agents. Candidiasis, an infection caused by fungal organisms in the *Candida* genus, often occurs as an opportunistic infection in immunocompromised individuals. Importantly, along with progressive disseminated histoplasmosis, cryptococcosis, and invasive aspergillosis, *Candida* infections are considered diagnostic hallmarks of acquired immunodeficiency syndrome (AIDS) (227,273). In addition to AIDS, improved life-sustaining technologies and aggressive anticancer therapy have contributed to the increasing incidence of candidiasis over the past two decades (99). In fact, *Candida* species have become the fourth leading cause of all nosocomial blood stream and urinary tract infections in U.S. hospitals (105).

The major etiological agent of candidiasis is *Candida albicans*, which constitutes 76% of clinical *Candida* isolates (272). *C. albicans* is a member of the human normal flora and can be isolated from the mouth (51), gastrointestinal tract (68), vagina (233), and skin (175). *C. albicans* has a broad clinical spectrum that can vary from superficial mucosal lesions (332) to life-threatening systemic or disseminated disease (174). The diverse clinical spectrum of infections, the limited number of cost-effective and safe antifungal agents (358), and the lack of ideal typing and diagnostic methods for *C. albicans* (166) have made it increasingly difficult to control candidiasis. Although the
number of available antifungal agents has increased over the past several years, the emergence of drug resistant strains and the relative toxicity of current antifungal therapy validate research aimed at discovering novel drug targets. Of particular interest is the mitochondrial organelle. Several therapeutic agents directed against the mitochondria of other human pathogens are effective (91), thus substantiating the utility of antimitochondrial therapeutics and the rationale behind mitochondrial research.

The mitochondria of *C. albicans* have been poorly characterized to date. The majority of information on mitochondrial structure, function, and genetics in fungi has been gained from research on several non-pathogenic organisms, including *Saccharomyces cerevisiae* (382). My dissertation has focused on several unresolved features of the *C. albicans* mitochondrial genome. One feature of mitochondrial organelles in general, is that they are dynamic with respect to both size and number throughout the cell cycle and in response to environmental conditions (21,153,354). One unique feature of *C. albicans* is that it is a polymorphic fungus which exhibits three different morphologies both in *vivo* and in *vivo*: yeast, pseudohyphae, and hyphae. Although several systemic fungal pathogens undergo morphogenesis, they are usually found as yeasts in humans (191). *C. albicans* is capable of existing in either form in the host and there has been much speculation on the role of hyphae in pathogenesis. Hyphal formation in *vivo*, as discussed below, can be induced by various growth conditions. I chose the yeast-to-hyphal transition to study differences in the mitochondrial genome copy number and changes in mitochondrial activity. The remainder of this introduction gives an overview of the current level of understanding of *C. albicans* mitochondria. I
will discuss clinical aspects of candidiasis, morphogenesis of *C. albicans*, and mitochondrial function and genetics as they pertain to my research topic.

**Clinical Aspects of Candidiasis**

In 1970, the National Nosocomial Infections Surveillance (NNIS) system was established to obtain data from U.S. hospitals regarding the type and incidence of nosocomial infections by use of standardized procedures (174). According to the NNIS, *Candida* species accounted for 72.1% of all fungal nosocomial infections between 1980 and 1990 (20). *C. albicans* is clearly the leading cause of candidiasis, representing 76% of all clinical *Candida* isolates (272). However, recent reports suggest that shifts have occurred in the distribution, and thus importance of non- *albicans* species of *Candida* (274,282,369). *C. glabrata, C. tropicalis, C. krusei, and C. lusitaniae* have become more frequently reported in association with infection (25). The mortality attributable to invasive *Candida* infections is quite impressive with crude mortality rates estimated at 50 to 60% in total (43,198), and as high as 73 to 90% in bone marrow transplant patients (237). In hopes of reducing the severity of disease, many epidemiological surveys have been undertaken to specify at-risk populations (7,174).

**Predisposing Factors to Candidiasis**

Multiple factors have been ascribed to increased susceptibility to *Candida* infections and have been thoroughly reviewed in the literature (259). As mentioned previously, *Candida* species can cause mucocutaneous and disseminated forms of candidiasis. Disseminated disease is usually associated with compromised immunity.
Important factors that induce immunosuppression in the host include aggressive chemotherapy (184) and corticosteroid treatment (198). Neutropenia, which may be induced by drugs or disease, is also a predisposing factor to disseminated candidiasis (364). Prolonged administration of antibacterial agents tends to promote the overgrowth of *Candida* sp. and can lead to a clinical manifestation of disease (190). Additional risk factors are those that provide a nidus of infection such as indwelling venous catheters (24,29) or invasive surgical procedures (43). Underlying disease is another risk factor as demonstrated by the high incidence of candidemia in patients with acute lymphocytic leukemia (307).

**Diagnosis of Candidiasis**

Early initiation of antifungal therapy is critical in reducing the mortality of disseminated candidiasis (7,154). The determination of systemic candidiasis is not a trivial consideration and in fact, has been problematic. Traditional diagnostic blood cultures (90) and antibody-based diagnostic schemes (98) are often times negative in candidiasis patients. In many cases, these two methods yield negative results despite histopathologic evidence of *Candida* invasion (163). In one study, 56% of necropsy proven cases were negative by blood culture (76). In addition, there is a poor correlation between systemic infection and candidemia. Candidemia does not always lead to systemic disease (360) and experimental animal models often show low levels of fungemia (167). Newer approaches for early and rapid detection of invasive candidiasis include monitoring the serum and/or urine levels of *Candida* cell-wall antigens, such as mannann/mannoproteins (251,279) and enolase (356), and *Candida* metabolites, such as
D-arabinitol (213). PCR-based methods to detect *Candida* DNA are also in the early stages of development (180). Clinical assessment of these various diagnostic tests has been performed, and though promising compared to blood culture, they tend to lack specificity and/or sensitivity (229).

**Anticandidal Drug Therapy**

Management and treatment of candidiasis rely primarily on the use of antifungal agents, both prophylactically and therapeutically. The current repertoire of antifungal drugs with systemic activity includes: amphotericin B, the azoles, and 5-fluorocytosine. Unfortunately, these drugs have several limitations including toxicity to the host and/or a rapid development of resistance by *Candida* (185). Amphotericin B is directed against ergosterol, a major component of the fungal plasma membrane. Ergosterol contributes to a variety of important cellular functions required for cellular growth and division (152,351). Amphotericin B is inserted into fungal membranes and functions by disruption of the membrane integrity (192). Although amphotericin B is historically the drug of choice for systemic candidiasis and other systemic fungal infections, this drug has important clinical limitations. Renal dysfunction and infusion-related toxicities are commonly associated with high dosage therapy (350), and drug-resistant strains of *Candida* species are increasingly reported (256).

5-fluorocytosine (5-FC) is another antifungal agent that is effective against *Candida*. 5-FC is transported into the cell by a cytosine permease and metabolized to form 5-fluorodeoxyuridine monophosphate and 5-fluorouridine triphosphate (192). The net result is disruption of both DNA and protein synthesis, respectively. Although quite
effective in susceptible yeast strains, the use of 5-FC is limited by a rapid development of resistance, particularly when used as a single agent (104). Natural resistance to 5-FC is also a concern because it occurs in up to 20% of *C. albicans* strains tested (335). 5-FC is most frequently used as adjunctive therapy with amphotericin B, however, toxicity of 5-FC remains a concern. The use of combination therapy has increased toxic effects due to 5-FC from less than 5% to 15-30% of patients (104). Conversely, the use of combination therapy reduces the optimal dose and toxicity levels of amphotericin B.

The azole antifungals have become important therapeutic agents for the treatment of candidiasis, owing to their relative safety, ease of delivery, and overall effectiveness (22,137). The azoles prevent the demethylation of lanosterol, an intermediate in ergosterol synthesis and are generally fungistatic agents (177). The azoles most commonly used for the treatment of disseminated candidiasis include: ketoconazole, fluconazole, and itraconazole (185). Fluconazole has become widely used for the treatment of AIDS-associated oropharyngeal candidiasis (9) and to prevent and treat candidiasis in neutropenic patients (217). The rise in popularity of fluconazole is likely due to a high oral bioavailability (69). The prophylactic use of fluconazole has also been shown to decrease the incidence of invasive candidiasis in patients receiving chemotherapy and bone marrow transplantation (126,322). Fluconazole is also effective against vaginal candidiasis in single-dose therapy (325). Itraconazole and voriconazole are also promising candidates, but their clinical usefulness is still under investigation. Unfortunately, concomitant with the widespread usage of azole antifungals has been the
emergence of drug resistant strains. *C. krusei* and *C. glabrata* are intrinsically resistant to fluconazole (260).

The development of fluconazole resistance is a complex issue. Many studies report increased fungal resistance in patients receiving high total cumulative doses of fluconazole (283), long-term therapy (238), and recent exposure to fluconazole (281). Conversely, one study revealed that fluconazole resistance can occur without prior exposure and furthermore, *C. albicans* developed resistance in several patients receiving large doses of fluconazole (355). In that study, *C. albicans* resistance to single dose treatment with fluconazole is also reported.

The limitations of current antifungal therapy have renewed interest not only in the development of more effective agents, but also in the identification of novel therapeutic targets. Ergosterol has been the primary target for drugs directed against fungal cell membranes. Recently, a new generation of antifungals directed at another essential membrane component, sphingolipids (5), have been discovered. Aureobasidin A is an inhibitor of sphingolipid synthesis. In a mouse model of systemic candidiasis, aureobasidin A is more effective than either amphotericin B or fluconazole (342). It was suggested that aureobasidin A is a strong inhibitor of expression of the ABC transporter/efflux pump, CDR2 (141). Increased expression of the *CDR* genes is correlated with azole resistance in *Candida* (296), which implies that aureobasidin A should be a good candidate for conjunctive therapy with fluconazole.
Morphogenesis

"Few fields of biological science based on so simple an observation, can have generated such a confused and contradictory literature as that of dimorphism in *Candida albicans*"- F.C. Odds (259).

*C. albicans* is a polymorphic fungus, as it can exist as a simple yeast form or undergo morphogenesis and produce filaments in the form of pseudohyphae and/or hyphae. Pseudohyphae are characterized by constrictions at septal junctions upon polar bud formation and tend to form in chains, whereas hyphae form parallel walls during apical extension and develop true septa. All forms are usually present in clinical lesions, which has led to considerable speculation and experimentation on the virulence role of hyphae in the pathogenesis of candidiasis (71).

The formation of hyphae in *C. albicans* occurs in response to several different *in vitro* mechanisms of induction. The properties and conditions supporting *in vitro* hyphal growth have been reviewed (259). In general, environmental stimuli such as temperature, pH, serum, CO₂, and nutrition, may induce a yeast-to-hyphal transition *in vitro* (248). The mechanisms behind hyphal production are enigmatic, which has led to the controversy surrounding hyphae as a virulence factor. Based on the current level of understanding of *C. albicans* morphogenesis, the role of hyphae in pathogenesis is inconclusive.

**Cell Biology and Physiology of Morphogenesis**

Insights into the process of morphogenesis in *C. albicans*, as well as many other filamentous fungi, have surprisingly come from information gained from wild-type cells
and not from mutants. In *C. albicans*, early processes in germinating cells are very similar to normal yeast production. Initiation of germination under appropriate growth conditions leads to an increase in the rate of volume growth of the mycelium identical to that of a budding yeast cell (148). The surface areas to volume ratios, however, are quite different with a much larger surface area in germlings. Implied in this finding is that initial hyphal growth is accompanied by the production of a thinner cell wall, which is in agreement with ultrastuctural analysis on *C. albicans* germ tubes (58,178). Cell-wall biosynthesis and cell extension occurs primarily at the tapered apical region (125,133). In growing hyphae, approximately 90% of wall extension occurs in the hyphal tip, whereas in budding yeasts only 70% occurs at the apex of the bud (331). Cytoplasmic content in growing hyphae remains constant, undergoes a steep rise in pH (334) and is accompanied by a migration of protoplasm from the parent cell to the hyphal apex (134). Initial hyphal elongation proceeds at a linear rate (45), but only the apical cells are metabolically active. Intercalary compartments remain uninucleate (136), largely vacuolated (134), and are arrested in the G1 phase of the cell cycle (136). Prior to the formation of secondary germ tubes and branches in mature *C. albicans* hyphae, cytoplasm is regenerated and growth proceeds at a logarithmic rate (135,148).

The induction of germination and hyphal production requires specific changes in the architecture of the cell wall. The exoskeleton of the fungal wall is composed of a complex network of polymers that imparts rigidity and determines the cell shape. The cell wall in *C. albicans* is composed of mannoproteins (20-30%), β1,3-linked glucans (25-35%), β1,6-linked glucans (35-45%), protein (5-15%), lipid (2-5%), and chitin (.6-
Cellular growth involves the formation of newly synthesized wall components in conjunction with the degradation or rearrangement of existing structures. The localization of secretory vesicles to the apical tip is required for hyphal elongation. The vesicles are highly refractive and can be seen by use of brightfield microscopy (38,157). Transport of the secretory vesicles from Golgi equivalents in *C. albicans*, requires actin microfilaments (379). Initiation of germination is accompanied by increased levels of actin transcripts (78,210,268). The role of cytoplasmic microtubules in morphogenesis is less clear. In one study, specific inhibition of microtubule formation by nocodazole in pre-germinated cells led to normal hyphal production (379). In another study, treatment of yeast cells with the anti-microtubule drug benomyl inhibited the formation of germ tubes and changed the distribution of actin filaments (3). These data indicate that *C. albicans* microtubules may be more important for the initiation of germination than for the production of mature hyphae. Microtubules and associated motor proteins, kinesins and dyneins, are involved in organelle localization and hyphal growth in several other filamentous fungi (88,142,156,372).

Information is limited on mechanisms of cell wall degradation and synthesis in *C. albicans*, but changes involving specific polysaccharides during germ tube formation have been reported. For example, the cell wall of germ tubes contains proportionately more β-1,3-linked glucans than either yeasts or mature hyphae (127). A three- to five-fold increase in cell wall chitin occurs immediately following germ tube formation (56). Similarly, studies on mRNA levels of chitin synthase genes during germination showed increased expression that peaked 1-2 hours after induction (61,248,339). Disruption of a
hyphal-specific chitin synthase gene \((CSH2)\) did not effect germination or virulence (130). The transcription of chitinase also demonstrates significant differences between yeast and hyphae. Transcription levels of two chitinase genes are greater during yeast cell growth versus hyphal growth (234). This implies that the restructuring of chitin is more important in yeast cell growth than in hyphal cells.

Several reports have implicated a role for sterols in hyphal formation. Free sterol content progressively increases during germination (240), and the total sterol content in mycelial lipids greatly exceeds that of yeasts (115). Sterol biosynthesis inhibitors, including the majority of azole antifungals, have also been shown to negatively effect germination (52,316). Several cell wall proteins are associated with morphogenesis in \(C.\ albicans\) (table 2), however, their role in hyphal production is unclear.

**Hyphae as a Virulence Factor**

A virulence factor, simply defined, is any factor produced by a fungus that increases virulence in the host. Virulence in \(C.\ albicans\) is a complex issue and appears to be the result of multiple factors (71). Many investigators have focused on \(C.\ albicans\) morphogenesis as a putative virulence characteristic. However, the evidence for hyphae as a virulence factor is unclear. For example, there is a general lack of agreement on the predominating form in either colonization or disease (259). In fact, both yeast and filamentous forms are usually present at sites of infection (51). These observations do not preclude the role of hyphae in pathogenesis. As described below, comparative studies with yeast and hyphal cells indicate that differences beyond cell type exist. In
many cases hyphae demonstrate increased adherence and invasive properties, which are characteristics involved in the pathogenesis of *C. albicans* (258).

**Adherence.** The ability of *C. albicans* to adhere to various surfaces and tissue/cell types has been intensely investigated and reviewed (51,71,259,270). Hyphal forms more readily adhere to human epithelia (194,326) and endothelia (289) than yeast cells. This hyphal property may be due to increased hydrophobicity as compared to yeast cells (254). Hydrophobic yeast cells show greater binding to epithelial cells than hydrophilic yeasts (93).

**Invasion.** Hyphae or the production of hyphae have been implicated in the invasion of host tissue. Several investigators have reported increased numbers of hyphal cells involved in both epithelial and endothelial penetration (96,158,289). Migration of *C. albicans* across the endothelial cell layer was proposed as a prerequisite for multi-organ involvement in disseminated candidiasis (196,381). If this hypothesis is correct, then increased hyphal penetration of endothelium suggests a mechanism for dissemination of *C. albicans*. There is little evidence to substantiate this conclusion.

**Thigmotropism.** Ordered hyphal growth in *C. albicans* is a characteristic that may facilitate tissue invasion. Two independent reports have demonstrated a helical growth pattern of *Candida* hyphae on a solid surface (179,313). In addition, *C. albicans* hyphae have been shown to grow by contact sensing, or thigmotropism (131,253,314). In these studies, hyphal extension proceeded along grooves and through pores on a solid membrane support. This property may allow *C. albicans* hyphae to penetrate some
tissues by following surface discontinuities and microscopic lesions. There is little evidence that demonstrates thigmotropism in vivo, although parallel hyphal penetration into intracellular spaces has been observed by ultrastructural analysis of oral infections (277) and vaginal tissue (261).

**Extracellular Enzymes.** Tissue/cellular damage is associated with the production of hyphae and is proposed to be enzyme-mediated (14). Both hyphal and yeast forms of *C. albicans* are capable of producing secreted aspartyl proteinases (SAP), which may participate in tissue invasion (246). SAP genes are differentially expressed between yeast and hyphal forms. A multigene family with at least nine known members (*SAPI* to *SAP9*) is encoded by *Candida* (235). *SAPs 4, 5, and 6* are expressed only in germ tubes in media containing serum as a source of nitrogen (160, 363). Gene disruption of these three related products yielded a strain with attenuated virulence in both guinea pig and mouse models of systemic candidiasis (295). Germination in the same triple deletion mutant was severely impaired. The significance of these data is unclear as it was not determined whether attenuated virulence was due to Sap enzyme deficiencies or abnormal hyphal production. The role of individual Sap isoenzymes in the virulence of *C. albicans* has not been clearly established. As discussed later, gene disruption experiments of putative virulence factors in *C. albicans* are often difficult to interpret.

Increased levels of another *Candida* putative virulence factor, extracellular phospholipase, are also associated with enhanced hyphal production (168). In this study, the levels of phospholipase production in several blood isolates of *C. albicans* were measured. These isolates were further analyzed for their ability to germinate and were
tested in a mouse model of disseminated candidiasis. A correlation was observed between increased phospholipase production, the ability to germinate, and increased mortality. Phospholipase, acid phosphatase and glucosaminidase activities were concentrated at the bud site of yeasts and the apical tips of germ tubes (351). The localization of these enzymes to regions of cellular growth most likely represents a role in structural changes required for cellular elongation and replication. This would explain why a correlation between increased phospholipase production and the ability to germinate was observed (168).

Genetics of Morphogenesis in *C. albicans*

Features of *C. albicans* that confounds genetic studies are that this fungus is diploid or polyploid and apparently lacks a sexual state (262). This property has not hindered genomic plasticity in this organism. Genomic plasticity in *C. albicans* is perhaps best demonstrated by extensive karyotypic polymorphism (291,292). Seven chromosomes have been ascribed to the *C. albicans* genome and each can vary in size as evidenced by pulsed-field electrophoretic analysis (220,221). The genetic basis of karyotype variation is unknown.

Historically, molecular biology and classical genetic analysis in this fungus have been hampered by the diploid state. The creation of mutants to address specific virulence factors (i.e., hyphal formation) relied upon non-specific mutagens and natural variants (table I). Differences in virulence were observed between mutants and wild-type strains in mouse models of candidiasis (71). However, a major caveat of using these mutants in virulence studies is that they lack genetic characterization. *C. albicans* molecular
methods are available that facilitate the creation of specific genetic mutations. Complementation (129), gene disruption (204) and transformation systems (189) have all been described and can be used to create genetically defined *C. albicans* mutants.

Table 1. *Candida albicans* hyphal mutants of non-defined specificity

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Characteristics</th>
<th>Virulence/model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B311V6</td>
<td>Non-hyphal variant</td>
<td>Attenuated/vaginitis</td>
<td>(326,362)</td>
</tr>
<tr>
<td>2. hOG301</td>
<td>Nitrosoguanidine-hyphae only</td>
<td>Non-pathogenic/systemic</td>
<td>(159,310)</td>
</tr>
<tr>
<td>3. CA2</td>
<td>Echinocandin resistant non-hyphal variant</td>
<td>Attenuated/vaginitis/systemic</td>
<td>(77)</td>
</tr>
<tr>
<td>4. MM2002</td>
<td>Antibody selected non-hyphal variant</td>
<td>Virulent/systemic</td>
<td>(293)</td>
</tr>
</tbody>
</table>

*Saccharomyces cerevisiae* As a Model to Understand *C. albicans* Morphogenesis

In efforts to determine whether the ability to form hyphae is important for virulence, several investigators have used *S. cerevisiae* as a model in which to identify *C. albicans* genes that may either enhance or suppress hyphal formation. This approach is based on the assumption that a similar mechanism of hyphal induction is shared between these two organisms. Diploid strains of *S. cerevisiae* undergo a yeast-to-pseudohyphal transition when nitrogen starved (121). In *Saccharomyces*, this transition depends on factors that participate in the mitogen-activated protein kinase (MAPK) pathway, including Ste7p, Ste11p, Ste12p and Ste20p (215). *Saccharomyces* mutants deficient in
any or a combination of these signal transduction gene products were defective in both mating and filamentous growth. Although *C. albicans* does not possess a complete mating pathway, this fungus has genetic elements that complement several mating defects in *Saccharomyces* (65,197,211,212,214,222,319,320). Sequential disruption (double-allelic knockout) of any of these mating gene homologs in *C. albicans* resulted in mutants with the inability to form hyphae under certain *in vitro* growth conditions (197,211,212,214). One fundamental difference between *S. cerevisiae* and *C. albicans* was that wild-type strains and gene-disrupted mutants of the latter formed hyphae in response to serum, whereas wild-type strains and mutants of *S. cerevisiae* did not. This observation implies important genetic differences in regulation, control and expression of filamentation between these two fungi.

The results of homologous gene studies yielded data that indicate further differences between *C. albicans* and *S. cerevisiae* filamentation pathways. Tup1, a general transcription repressor in *Saccharomyces* (187), participates in filamentation in both *S. cerevisiae* and *C. albicans* (39). Whereas the mutant *C. albicans* strain produced filaments on all media, the analogous mutant of *Saccharomyces* had a markedly reduced ability to produce pseudohyphae in a nitrogen-deprived medium. The point is, disruption of the *TUP1* gene yielded opposite effects in *C. albicans* as compared to *S. cerevisiae*. Although *C. albicans* shares many gene homologs with *S. cerevisiae*, the *TUP1* studies indicate that there are important differences in morphogenesis of these fungi.
Development of Agerminative Mutants

Efforts to develop agerminative *C. albicans* mutants have prompted investigation into the genetic basis of the residual filament formation exhibited by *Candida* strains deficient in MAPK gene homologs of *S. cerevisiae*. As mentioned above, whereas these *C. albicans* mutants were non-filamentous on serum-free media, they still formed hyphae in response to serum (197,211,214). Recently, in independent studies, Lo, et al. (216) and Stoldt, et al. (336) demonstrated that another putative transcriptional regulator, Efg1p, plays a role in hyphal formation. *EFG1* is a *C. albicans* gene homolog of *PHD1*, a *S. cerevisiae* pseudohyphal regulator (120). Either deletion of the gene in *C. albicans* (216) or reduced expression under an inducible promoter (336) yielded a pseudohyphal phenotype, rather than hyphae, in response to serum. Disruption of both *EFG1* and a MAPK gene homolog (*CPH1*) resulted in a *C. albicans* mutant (HLC54) that was incapable of hyphal formation under several different conditions, including a serum-containing medium (216). This finding is provocative in that it may provide insight into potential molecular switch mechanisms for morphogenesis. Unfortunately, the specific regulatory functions of the putative transcription factors Efg1p and Cph1p are unknown. If these factors affect multiple genes, the inability of a double mutant to form hyphae may be only one of several defects.

In attempts to address the question of whether the ability to form hyphae is a virulence factor, Lo et al. (216) designed experiments to test the pathogenicity of the HLC54 double mutant in a mouse model of disseminated candidiasis. The results of survival data indicated that HLC54 was less virulent than both wild-type *C. albicans* and
mutants lacking only one of the two transcription factors (Cphlp or Efglp). On the basis of these data, the authors concluded that the ability of *C. albicans* to switch from yeast to the filamentous form was required for virulence. However, the results of their virulence studies on the heterozygous mutants, and discussion by the authors, support the suggestion that Cphlp and Efglp transcription factors affect multiple genes. The *EFG1/efg1* heterozygotes, which are capable of normal hyphal production *in vitro* (20% serum), were less virulent than the *EFG1/EFG1* homozygote. This indicates that *EFG1* may participate in a function that is not linked to cellular morphogenesis, but that it influences virulence nevertheless. As an alternative explanation, the *EFG1/efg1* heterozygote may not produce normal hyphae *in vivo*, but this was not determined. In agreement with the alternative explanation, Stoldt, et al. (336) showed an abnormal yeast/pseudohyphal form at a low *EFG1* expression level. This indicates that in addition to defective hyphal production, the yeast forms may not have been normal. This point is further demonstrated by studies on the gene encoding CaCla4p, a putative serine/threonine protein kinase (212) similar to Ste20p. *C. albicans* strains deficient in CaCla4p produced truncated germ tubes in the presence of serum and showed reduced virulence in mice. However, yeast cells produced in this strain were often multibudded and multinucleated (212). This behavior of yeasts during cellular division indicates a defect in cytokinesis and is not typical of wild-type yeast cells. Deletion of another MAPK gene in *C. albicans*, *MKCI*, has also been linked to deficiencies in cell integrity and attenuated virulence (85), and not to hyphal production. Although the Lo, et al. (216) mutants approximate *in vitro* growth rates of the wild-type strain, this criterion does not
preclude important yeast cell wall alterations that could account for the reduced virulence of the double mutant. Lo, et al. (216) concluded that the "switch" from yeast to hyphal morphogenesis, and not necessarily the form of the fungus, is important for virulence. Conversely, the \textit{EFG1/efg1} heterozygote underwent the switch, but were less virulent (216), and others found that \textit{HST7} and \textit{CST20} deletion mutants produced hyphae \textit{in vivo}, but were less virulent than the wild-type (211). Furthermore, deletion of a gene coding for an integrin-like protein of \textit{C. albicans} (Int1p) yielded very similar results to \textit{HST7} and \textit{CST20} disruption (111). The \textit{int1/int1} mutant exhibited reduced hyphal production under several \textit{in vitro} growth conditions and was less virulent than the wild-type strain. This homozygous mutant was capable of normal hyphal production when grown in serum-containing media. This indicates that the morphological switch is not an independent virulence factor.

The interpretation of \textit{in vivo} experiments on virulence of the HLC54 mutant was further complicated by the choice of control strains. In the genetic analysis of the mutant, Efg1p function was confirmed by transformation of HLC54 with a functional \textit{EFG1} gene, and subsequent restoration (strain HLC84) of the original filamentous phenotype. This critical revertant control was not used, however, in the mouse virulence studies.

Although hyphal growth is generally regarded as an important virulence trait of \textit{C. albicans}, mutants expressing predominantly filamentous phenotypes are not more virulent than wild-type strains. In fact, the previously mentioned \textit{TUP1} deletion mutants were less virulent in a mouse model of disseminated candidiasis (39). In addition to \textit{TUP1}, the disruption of a serine/threonine kinase gene, \textit{SNF1}, yielded an enhanced
hyphal phenotype (271). In *S. cerevisiae*, Snf1p interacts with the Tup1p repressor complex in glucose repression (378) and the RAS-cyclic AMP signal transduction pathway (347). *C. albicans* SNFL deletion mutants did not demonstrate increased virulence (271).

As pointed out by Lo et al. (216), the defined genetics of *S. cerevisiae* have been useful in providing insights into genetic aspects of morphogenesis of *C. albicans*. As we indicate here, there are limitations to conclusions that can be drawn from comparisons between these two species. In the past, the rationale for use of *Saccharomyces* as a model system to study the genetics of *C. albicans* was based primarily on the difficulties of working with the *Candida* diploid genome. Molecular techniques are now available for use in *C. albicans* that specifically address the complex genetic system of this organism. For example, methods for sequential gene disruption in *S. cerevisiae* (4) have been adapted for use in *C. albicans* (101). Importantly, such techniques allow studies to be done directly on *C. albicans*.

Conclusions

The search for the genetic basis of polymorphism in *C. albicans* has led to important recent discoveries concerning the basic molecular biology of this organism (table 2), including the initial characterization of putative transcription factors that affect hyphal formation. Several *S. cerevisiae* gene homologs are clearly involved in *C. albicans* morphogenesis. These elegant molecular studies, although fascinating, have not resolved the issue of hyphae formation and virulence in *C. albicans*. The multitude of significant differences between *C. albicans* and *S. cerevisiae*, including pathogenicity,
warrants direct studies on *C. albicans*. Revealing the unique features of the *C. albicans* genome will provide information about how this microorganism is able both to colonize and cause disease in humans.

Table 2. Morphogenesis associated genes in *Candida albicans*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product/Function</th>
<th>Deletion Phenotype</th>
<th>Virulence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <strong>ECE1</strong></td>
<td>H specific antigen</td>
<td>Normal</td>
<td>N.D.</td>
<td>(33)</td>
</tr>
<tr>
<td>2. <strong>PHR1/PHR2</strong></td>
<td>pH-regulated glycoprotein</td>
<td>Defective Y and H</td>
<td>Attenuated</td>
<td>(116,247,297)</td>
</tr>
<tr>
<td>3. <strong>PRA1</strong></td>
<td>pH-regulated H antigen</td>
<td>Defective H</td>
<td>N.D.</td>
<td>(39)</td>
</tr>
<tr>
<td>4. <strong>HYR1</strong></td>
<td>Cell wall glycoprotein</td>
<td>Normal</td>
<td>N.D.</td>
<td>(17)</td>
</tr>
<tr>
<td>5. <strong>CaRSR1</strong></td>
<td>Ras-related protein</td>
<td>Defective Y and H</td>
<td>Attenuated</td>
<td>(373)</td>
</tr>
<tr>
<td>6. <strong>KEX2</strong></td>
<td>proprotein convertase</td>
<td>Defective H</td>
<td>N.D.</td>
<td>(252)</td>
</tr>
<tr>
<td>7. <strong>PKC1</strong></td>
<td>protein kinase C</td>
<td>Defective Y and H increased hyphae</td>
<td>N.D.</td>
<td>(269)</td>
</tr>
<tr>
<td>8. <strong>SNF1</strong></td>
<td>ser/thr prot. kinase transcription factor?</td>
<td>Normal</td>
<td>N.D.</td>
<td>(271)</td>
</tr>
<tr>
<td>9. <strong>RBF1</strong></td>
<td>MAPK</td>
<td>Defective H only</td>
<td>Attenuated</td>
<td>(169,170)</td>
</tr>
<tr>
<td>10. <strong>MKC1</strong></td>
<td>MAPK</td>
<td>Defective Y and H</td>
<td>Attenuated</td>
<td>(85)</td>
</tr>
<tr>
<td>11. <strong>INT1</strong></td>
<td>integrin-like protein</td>
<td>Defective H</td>
<td>Attenuated</td>
<td>(110,111)</td>
</tr>
<tr>
<td>12. <strong>HST7</strong></td>
<td>MAPK</td>
<td>Defective H</td>
<td>N.D.</td>
<td>(211)</td>
</tr>
<tr>
<td>13. <strong>CPH1</strong></td>
<td>MAPK</td>
<td>Defective H</td>
<td>Attenuated</td>
<td>(216,222)</td>
</tr>
<tr>
<td>14. <strong>CST20</strong></td>
<td>MAPK</td>
<td>Defective H</td>
<td>Attenuated</td>
<td>(65,211,212)</td>
</tr>
<tr>
<td>15. <strong>EFG1</strong></td>
<td>transcription factor?</td>
<td>Defective H hyphae only</td>
<td>Attenuated</td>
<td>(216,336)</td>
</tr>
<tr>
<td>16. <strong>TUP1</strong></td>
<td>transcription repressor</td>
<td></td>
<td>Attenuated</td>
<td>(39)</td>
</tr>
</tbody>
</table>
Mitochondria

As mentioned previously, the central topic of this thesis concerns *C. albicans* mitochondrial genome copy number changes during a yeast to hyphal transition. An understanding of the importance of mitochondria in the biology of *C. albicans* necessitates a review of knowledge gained from non-*Candida* systems.

Mitochondria are highly variable organelles in regard to size, shape, chemical composition and function. Mitochondria primarily serve as a site for respiration and oxidative phosphorylation, however several other functions have been attributed to these diverse organelles. They also synthesize lipids, heme, amino acids, nucleotides, and they mediate the intracellular homeostasis of inorganic ions (348). Although mitochondria contain an independently replicating genome, distinct from that of the nucleus, the majority of mitochondrial proteins are encoded by nuclear genes and are transported to the organelle (374). Studies on petite mutants in *S. cerevisiae*, deficient in respiration and mitochondrial DNA, indicate that function of an intact mitochondrial genome is required for the development of a respiration-competent organelle (16).

Structure

Mitochondria are membrane bound organelles consisting of an outer and inner membrane. Both membranes are composed primarily of proteins and lipids. Phosphatidyl choline, phosphatidyl ethanolamine, and cardiolipin represent the majority of mitochondrial lipids (143). Detailed lipid analysis has revealed differences between the outer and inner membrane. The outer membrane contains ergosterol whereas the
inner membrane has a higher level of cardiolipin and no ergosterol (173). The differences in protein composition of these membranes are attributed to the presence of functional enzyme components. The outer membrane contains proteins involved in the transport of macromolecules and lipid biosynthesis (305). The inner membrane contains proteins required for oxidative phosphorylation including the four respiratory complexes and the ATP synthetase (305). The inner matrix contains the mitochondrial genome and protein synthesis machinery (139).

**Biogenesis/Protein Import**

Mitochondria proliferate by growth and division of pre-existing organelles (374). During cellular division, the faithful segregation of mitochondria and mitochondrial DNA into daughter cells is essential for cell viability (16). The process of mitochondrial inheritance requires both biogenesis of membranes and cytoskeletal involvement during cell division. Protein import into mitochondria is the major mechanism of mitochondrial biogenesis (299). Most cytoplasmic proteins destined for transport into the mitochondrial matrix are synthesized with N-terminal amphiphilic targeting sequences (19). The loosely folded precursor protein binds to the mitochondrial surface, a process mediated by mitochondrial surface receptors (249). Protein import into the matrix space requires the function of the mitochondrial ATPase, hsp70 chaperonin (155) and the outer membrane transporter ISP42 (18,353). Hsp70 acts in conjunction with the Tim44 subunit of the inner membrane import complex, and the nucleotide exchange factor mGrpE (183,300). Once the protein has reached the matrix, its targeting sequence is removed by
a series of matrix enzymes (MAS1, MAS2, and hsp60) and the mature polypeptide is folded into its native conformation (113,306).

Mitochondrial Behavior

The behavior of fungal organelles during cellular growth and division has been studied extensively. Controversies exist with respect to the number of mitochondria per yeast cell and their morphology. Early descriptions range from numerous ovoid mitochondria to a single branched mitochondrion, based on electron microscopic imaging (17,153,348,373). Evidence for the presence of a single mitochondrion was obtained by reconstruction of serial sections of entire *S. cerevisiae* yeast cells (153). Further mitochondrial analysis has revealed that yeast mitochondria are dynamic in both structure and number in response to environmental changes (193,333,354), including anaerobic growth (73). In addition to *S. cerevisiae*, serial section electron microscopic reconstructions have been performed on both *Schizosaccharomyces pombe* and *C. albicans* (182,343), indicating that mitochondria size and number in these fungi are also dynamic.

Three-dimensional reconstruction studies from multiple serial sections of whole *C. albicans* cells demonstrated that the mitochondria are dynamic during both cell division and germ tube formation (343). In this study, mitochondrial behavior in three isolates of *C. albicans* was compared at different stages of the cell cycle. The progression of the cell cycle was correlated with the extent of bud emergence and spindle development in the nucleus. Mitochondrial fusion and division events were dependent on the stage in the cell cycle and not on the growth or increase in volume of mitochondria.
The mitochondria fused into a single giant organelle upon bud formation and fragmented during mitosis. Coalescence occurred again prior to cytokinesis and fragmented shortly thereafter. The ratio of organelle volume to total cell volume remained relatively constant. The estimated numbers of mitochondria varied between 1-4 in strain NUM678 and 2-10 in strain IAM4966, indicating that there are strain to strain differences. Similarly, mitochondrial behavior during morphogenesis demonstrated the formation of a giant mitochondrion prior to germ tube formation, which fragmented during mitosis. Mitochondrial numbers in germinating cells varied between 1 and 7 in strain IAM4966. The average percentage of cell volume occupied by mitochondria was between 6 and 13% which is similar to data reported for the yeasts *Pityrosporum ovale* (186) and *S. cerevisiae* (140).

The dynamic behavior of mitochondria in both *C. albicans* yeast and hyphae has also been observed by fluorescence microscopy (13,171). Mitochondria were fluorescently stained with 2-(4-dimethlaminostyryl)-1-methylpyridinium iodide (DASPMI), which preferentially stains mitochondria based on the electron potential of the membrane (30). In contrast with data obtained from reconstruction methods, Ito-Kuwa et al., (171) reported the presence of a single branched mitochondrion that divided only upon cytokinesis. In addition, there were no cell cycle-related morphological changes of mitochondria which is in direct agreement with studies on *C. utilis* and *C. tropicalis* (75). The authors concluded that the form and number of mitochondria of *C. albicans* are primarily influenced by the physiological state of growth, comparable to mitochondrial behavior in the basidiomycetous yeast *Bullera alba* (344). Similar
observations were made for the yeast-to-hyphae transition of *C. albicans* (13) of which only a single mitochondrion enters the emerging germ tube. It is possible that the differences observed between serial section analysis and fluorescence microscopy are due to differences in the *C. albicans* strains chosen for study. Differences may have also been observed due to experimental methods. For example, in the fluorescence studies an epifluorescence microscope was used to generate two-dimensional images of the mitochondria, whereas three-dimensional analysis was used in the serial-section studies. Confocal-scanning laser microscopy techniques have since been used to construct three-dimensional models of mitochondria in *S. cerevisiae* (354), but not in *C. albicans*.

The requirement of mitochondria for yeast cell viability, in conjunction with observed behavioral changes during cellular growth and differentiation has brought attention to the process of mitochondrial inheritance. Several studies have addressed the question of whether mitochondrial mobility patterns are linked to the cell cycle. Bud formation and germ tube formation are highly polarized processes in several fungal species (60,121) including *C. albicans* (84,132). Prior to bud formation, mitochondria localize to the cell cortex (153) and are transported to the emerging bud during the G1-S phase of the cell cycle (333) suggesting that mitochondrial inheritance is tightly coordinated with the cell cycle. Both genetic and biochemical analyses suggest that this polarized transport of mitochondria to the bud is an active process that is actin-dependent (100,149,318). An intermediate filament-like protein, MDM1p has also been associated with mitochondrial inheritance (230,232). Temperature dependent MDM1p deficient mutants showed defects in transfer of mitochondria and nuclei into developing buds of
yeast cells at nonpermissive temperatures. Two additional actin-associated proteins, Mdm12p and Mdm20p are also required for mitochondrial inheritance (31,149). Yeast cells that failed to receive a mitochondrial compartment did not separate from the mother cell and were unable to produce buds (73,140,184,334). Further evidence that mitochondrial inheritance is linked to the cell cycle is provided by studies on a serine/threonine phosphatase, PTC1p in *S. cerevisiae* (286). Mutants deficient in *PTC1* displayed a delayed progression of mitochondrial inheritance. Changes in the actin cytoskeleton were not observed and nuclear migration appeared normal. Thus, the timing of mitochondrial transport also appears to be actively regulated and coordinated with the cell cycle.

**Respiration in *C. albicans***

The biochemical properties of mitochondria as they relate to oxidative phosphorylation and respiration, and the elucidation of numerous metabolic pathways have been well characterized and extensively reviewed (139,348). In addition, the biochemical characterization of isolated *S. cerevisiae* mitochondria has been well-defined (382). The biochemical properties of mitochondria and respiration in *C. albicans* have been studied to some extent with preparations of crude mitochondrial extracts (259,377). *C. albicans* contains elements of the major respiratory enzyme complexes (245) such as NADH dehydrogenase (36,80), succinate dehydrogenase (245,377), cytochrome oxidase and electron transport chain subunits cytochromes b, c, a and aa3 (2,2,377).

The cytochrome pathway in fungi appears to be the major mode of electron transport (139,348). In addition to this classical cytochrome pathway, an alternative
pathway of electron transport has also been described in fungi (6). Electron transport via the cytochrome pathway is blocked by either cyanide, azide, or high levels of carbon monoxide. The alternative cyanide-insensitive respiration pathway is characterized by inhibition with either salicyl hydroxamate (SHAM) or azide. *C. albicans* possesses a cyanide-insensitive alternative respiratory pathway (11,199,200,357), which can be expressed in both yeast and hyphal cells (206,312). Accordingly, in the presence of cyanide, respiration of *C. albicans* cells is inhibited by SHAM (199,311). Studies on respiration deficient mutants in *C. albicans* indicates that the most likely branch point for the alternative pathway occurs between coenzyme Q and cytochrome b (11,199).

Growth conditions that favor the expression of the alternative oxidase in *C. albicans* remain undefined or are controversial. In one study, inhibition of electron transfer to complex IV by cyanide initiated the expression of the alternate oxidase in both yeast and hyphae (311). The initial addition of cyanide immediately reduced respiration in cells, but fully recovered to normal levels within 20 minutes. Subsequent addition of SHAM inhibited respiration. Other investigators, however, were only able to reproduce this phenomenon in aged yeast cells and not in hyphal cells or growing yeasts (10,11). Although the alternative pathway allows respiration to continue in the presence of cyanide, the cyanide-sensitive pathway is required for growth. The role of the alternate oxidase in the growth and development of *C. albicans* has not been determined.

Several lines of evidence indicate that there are differences in respiratory requirements between yeasts and hyphae. Early studies on morphogenesis indicated that respiratory activity in yeast cells declined upon addition to a hyphae-inducing medium
and continued throughout germination (375). In the presence of glucose, *C. albicans* hyphae have been shown to produce more ethanol, evolve less CO₂, and consume less oxygen than yeast cells, thus indicating an abrupt change from an aerobic to a fermentative metabolism (206).

**Respiration Deficient Mutants**

The generation of mitochondrial mutants in yeasts has contributed enormously to the understanding of the function of mitochondria, mitochondrial biogenesis, and genetic characterization of the mitochondrial genome. The original description of mitochondrial mutants came from studies on *S. cerevisiae* (94). Mutations in mitochondrial respiration, oxidative phosphorylation, and protein synthesis were induced by growth in the presence of acriflavine, which resulted in the occurrence of 'petite colonie' or cytoplasmic petite mutants. By definition, petites must be grown on a fermentable carbon source. On a medium containing 0.1% glucose and 2% glycerol, wild-type cells form large colonies, whereas the colonies formed by petite mutants are small (118). Petite mutant cells use glucose as the carbon source and they are incapable of respiring the glycerol. Further studies on acriflavine-induced mutants have demonstrated that the observed mitochondrial deficiencies result from large deletions of the mitochondrial genome (97). In addition to nucleic acid mutations, acriflavine inhibits the synthesis of cytochrome components of the respiratory system during aerobic growth, which most likely precedes mutagenesis (47). Acriflavine-induced cytochrome inhibition is the result of blockage in mitochondrial protein synthesis and is similar in mechanism to chloramphenicol (188).
Growth of *S. cerevisiae* petite mutants is supported by fermentation in the absence of a functional mitochondrial oxidative phosphorylation system (348).

Numerous attempts have been made to generate petite mutants in *C. albicans*. *C. albicans* was described as a petite-negative yeast in 1964 (47). Since then, acriflavine-induced petite mutants have been described (370). These *C. albicans* respiration-deficient mutants approximate *S. cerevisiae* cytoplasmic petites, namely reduced oxygen consumption and growth rates. Several *C. albicans* petite mutants also demonstrated the loss of cytochrome aa3 (12), which is a characteristic of *S. cerevisiae* petites (118). It is clear that these *C. albicans* acriflavine-induced mutants share features of *S. cerevisiae* petites and have impaired mitochondrial function. However, their classification as petite mutants is controversial. Others have reported *C. albicans* respiration deficient mutants, but had difficulties in obtaining petites (2).

The rigorous genetic screening used to define mitochondrial DNA deletions in *S. cerevisiae* cytoplasmic petite mutants was not used to define the *C. albicans* petite mutants. For example, specific mutational events in the *C. albicans* mitochondrial genome were not documented (370) and respiration deficiency in these mutants most likely stems from lesions in nuclear genes (284). Several nuclear defects in *S. cerevisiae* are associated with mitochondrial dysfunction (97). Cytoplasmic petites in *S. cerevisiae* also display a loss of functional cytochromes aa3, b, and c (107), whereas the *C. albicans* petite mutants were only deficient in cytochrome aa3 (376) or displayed the complete cytochrome spectrum (12). *C. albicans* respiration deficient mutants not referred to as petite mutants (reduced oxygen consumption and growth rate) were also deficient in
cytochrome aa₃ and additionally lacked cytochrome b (2). Furthermore, *S. cerevisiae* mitochondrial petite mutants are classified into vegetative (non-mendelian) or segregational (mendelian) petites based on mitochondrial DNA inheritance patterns (118), but this kind of analysis was not done on the *C. albicans* mutants (12). Based on the original description of petite mutants in *S. cerevisiae* (94), *C. albicans* appears to be a petite negative yeast.

**Mitochondrial Genetics**

An interesting feature of mitochondria is the requirement of an independently replicating genome in mitochondria of some eukaryotes, and the complete absence of a mitochondrial genome in others (118). This feature raises questions concerning the necessity for, and maintenance of a mitochondrial genome. The mitochondrial genome contains genes for several essential mitochondrial proteins, but the nuclear genome of the majority of eukaryotes contains genes for over 90% of the mitochondrial proteins (348). In fact, indirect evidence suggests that organisms are evolving away from the requirement of a mitochondrial genome. Complete loss of mitochondrial DNA has occurred in several eukaryotes, but these organisms retain an energy generating mitochondrial-like organelle called a hydrogenosome (32,265). Similar to mitochondria, hydrogenosomes have a double-membrane envelope, divide autonomously by fission, import proteins post-translationally, and can produce ATP by substrate-level phosphorylation (83). In organisms with hydrogenosomes, genes encoding three mitochondrial heat shock proteins (Hsp10, Hsp60, Hsp70) are found on nuclear chromosomes (46), which suggests a mitochondrial origin. Hydrogenosomes are
characteristic to several anaerobic eukaryotes including many ciliates and protists (265). The hydrogenosomes in these organisms resemble mitochondria of anaerobically grown \textit{S. cerevisiae}. Anaerobic growth of \textit{S. cerevisiae} on glucose causes a reduction in respiratory cytochromes and prevents the formation of mitochondrial unsaturated fatty acids and ergosterol (266). The mitochondria of these \textit{S. cerevisiae} lipid-depleted cells are inactive in protein synthesis, lack mitochondrial DNA, and are incapable of converting to aerobic respiration (118).

Mechanisms for the retention and requirement of mitochondrial DNA in the majority of eukaryotes have not been well defined. The hydrophobic nature of several mitochondrial proteins may well necessitate an intra-organellar site of synthesis, thus obviating a need for transport from the aqueous environment of the cytoplasm to the mitochondrion (66,67). A prediction is that extremely hydrophobic proteins required for mitochondrial function must be encoded internally in the mitochondrial genome (138). The problem with this hypothesis is that the highly hydrophobic ATPase subunit 9 is encoded by a nuclear gene in \textit{Neurospora crassa} and is transported to the mitochondria (59), which requires transport across the aqueous cytoplasm before assembly in the mitochondrial inner membrane. This indicates that hydrophobicity of mitochondrial encoded proteins is not the only requirement for the retention of a mitochondrial genome.

Mitochondrial Genome Organization and Gene Content

The relative importance of mitochondrial DNA has led to considerable efforts to sequence entire mitochondrial genomes in several species including humans (8).
Mitochondrial sequence analysis in humans has uncovered numerous genetic disorders resulting from deficiencies or mutations in mitochondrial genes (207,380). The fact that mitochondrial DNA has much less redundancy than the nuclear genome and much higher information density (an equivalent complement of essential nuclear genes would span 1-2 million base pairs), makes it an excellent target for the expression of mutants. However, not all mitochondrial mutants are viable, which has led to interest in identification of mitochondrial genes.

Several yeast mitochondrial genomes have been sequenced in their entirety including: *Hansenula wingei*, S. *douglasii*, *Schizosaccharomyces pombe*, S. *cerevisiae*, and S. *uvarum* (53,54,82,267,308). This list is not exhaustive and efforts are currently in progress to obtain representative mitochondrial genome sequences across the entire fungal taxa (267). Analysis of mitochondrial DNA sequences has revealed several genes ubiquitous to fungi. The majority of fungal mitochondrial DNA contains genes encoding large and small ribosomal RNAs, a complete spectrum of tRNAs, three subunits of cytochrome c oxidase, three subunits of ATPase, and apocytochrome b (119). Differences have been observed between yeasts and filamentous fungi. Mitochondrial DNA in several yeast species contains the VAR1 gene, encoding a mitochondrial ribosomal protein (82,119,308). Filamentous fungi generally contain mitochondrial genes encoding subunits of NADH dehydrogenase (44,82,308). NADH dehydrogenase genes are an unusual component of the yeast *H. wingei*, and are not found in *S. cerevisiae* (308). Size differences in fungal mitochondria are attributed to the presence/absence of introns and to the length of intergenic regions (82,371). Introns in S.
cerevisiae contain short G + C rich sequences (GC clusters), which are mobile elements and hot spots for recombination in the mitochondrial genome (359). In contrast to vertebrate mitochondrial genomes, which generally have G+C contents greater than 40%, the G+C content of most yeast species is around 18% (82), which indicates that G+C composition may be of regulatory importance in yeasts.

The structural organization of genes in mitochondrial genomes is also highly variable (371). One interesting structural feature of these genomes is the presence of amphimers or inverted repeats. Inverted repeat sequences are common in chloroplast genomes, but are unusual in mitochondrial DNA (275). In fungi, inverted repeats have been reported in Achlya, Phythium, Agaricus, Agrocybe, C. albicans, and Kloeckera africana (64,151,162,236,368). Special cases of multiple inverted repeats have been noted in the mitochondria of petite mutants in S. cerevisiae, but not in their wild-type parent strains (276). The function of inverted repeats in mitochondrial DNA is unclear, but others have proposed that the structure of inverted repeats may be the only form in which a duplicated segment can be stably retained in an amphimeric genome (37). If duplicated segments of DNA are present as tandem repeats in an amphimeric genome, homologous recombination would tend to eliminate one copy (275).

The majority of mitochondrial genomes appear to be circular (26), but linear forms are also known to exist in fungi (108). The mechanism for mitochondrial replication is under investigation, and the rolling circle method may occur in yeasts (223). Linear mitochondrial DNA molecules may be intermediates of replication (17,26,27) rather than an artifact due to mechanical shearing during DNA isolation (26),
but this point has not been resolved. In many cases, the linear form of mtDNA is larger than the circular forms, which would tend to rule out a broken circle theory (26). Furthermore, several fungi contain linear mitochondrial DNA molecules with hairpin structures at both termini, suggesting that these structures are present to preserve the ends of the linear genome (86). Pulsed-field electrophoretic methods often reveal the presence of both linear and circular forms of mitochondrial DNA (26), which indicates that both forms are important in both replication and function (17,27).

Mitochondrial genomes are organized into electron dense areas referred to as nucleoids or nuclei (203). Within nucleoids, mitochondrial DNA is associated with arginine/lysin-rich proteins, which resembles nuclear histones (55). The number of mitochondrial genomes per nucleoid varies between one and eight (371), but most mitochondria contain one nucleoid (203). Mitochondrial genome copy number per cell is also variable. The *S. cerevisiae* mitochondrial genome copy number is approximately 50 per haploid cell, which is based on the amount of mitochondrial DNA relative to total DNA (13.5±1.3%) (140). However, the amount of mitochondrial DNA per cell has been reported to vary between 5 and 25% of total DNA (371). By extrapolation, the number of mitochondrial genomes in *S. cerevisiae* may vary between 17 and 87.

The timing of mitochondrial replication in relation to the cell cycle appears to be species dependent. In *S. cerevisiae*, replication of mitochondrial DNA takes place continuously throughout the cell cycle (202,241,366). In contrast, mitochondrial DNA synthesis is periodic during synchronous growth in *S. pombe* and *S. lactis*, and occurs at a different time than nuclear DNA replication (79,323). The entire mitochondrial DNA
population may replicate synchronously in an *S. cerevisiae* culture, providing further evidence of nuclear control of mitochondrial DNA replication in this fungus (70).

**Mitochondrial DNA in *C. albicans***

The *C. albicans* mitochondrial genome is circular with a molecular size of 40 kb (309,367,368). The base composition of the genome consists of a G+C content of 38.2%, which is similar to the 33% G+C content of the nuclear genome (368). The G+C content is exceedingly higher than the 18% G+C content of *S. cerevisiae* mitochondrial DNA and more similar to the 40% G+C content of vertebrates (82). Extensive restriction map analysis of *C. albicans* mitochondrial DNA has been done by use of an *EcoRI* library of the circular genome (368). The use of the *EcoRI* fragments as DNA probes, revealed the presence of a large inverted repeat in the mitochondrial genome (309,368). The unique feature of the inverted repeat is that it does not contain rDNA, which is a characteristic of the majority of mitochondria and chloroplasts containing inverted repeats (275). Hybridization analysis using *S. cerevisiae* mitochondrial gene probes indicates that the inverted repeat contains the subunit 3 of cytochrome oxidase (309). However, the gene content of the majority of the inverted repeat remains unknown. The map position of other *C. albicans* mitochondrial genes was also determined by using known *S. cerevisiae* genes as probes (see figure 1) (309). The sequence of several mitochondrial genes in *C. albicans* has been revealed by efforts towards sequencing the entire genome, but their position on the mitochondrial map has not been determined.
other *C. albicans* mitochondrial genes was also determined by using known *S. cerevisiae* genes as probes (see figure 1) (309). The sequence of several mitochondrial genes in *C. albicans* has been revealed by efforts towards sequencing the entire genome, but their position on the mitochondrial map has not been determined.

Figure 1. Map of the 40 kb circular mitochondrial genome of *C. albicans*. The arrow-tipped arcs represent the location of the inverted repeat (with permission (284)
that the *C. albicans* mitochondrial genome is highly conserved between strains and therefore, may represent a novel therapeutic target. In an extension of these findings, the presence of species-specific regions in the mitochondrial DNA of *C. albicans* was proposed by several investigators (102, 243, 263). Miyakawa and Mabuchi (243) reported the cloning of a species-specific DNA region that originated from the mitochondrial genome. Further analysis of their clone, EO3, revealed that the DNA fragment mapped to a portion of the inverted repeat of the mitochondrial genome (244). RFLPs exist in this portion of the inverted repeat, classified as L, M, and S type strains. Sequence analysis indicated 50-60 bp differences at two positions in the EO3 fragment. The authors chose to emphasize the utility of a species-specific fragment in epidemiological studies and clinical identification of *C. albicans*. Perhaps more importantly, the presence of a species-specific DNA fragment originating from the inverted repeat of the mitochondrial genome, signifies that unique sequences occur in *C. albicans* mitochondrial DNA. Unfortunately, the authors did not investigate what genes, if any, are contained in this portion of the genome.

**Mitochondrial Drug Targets**

The final consideration concerns the potential exploitation of the mitochondria as an antimicrobial target, which is an area of research that has received very little attention. As demonstrated above, several features of mitochondria are conserved across the taxa (348, 374) and the majority of similarities are due to the common respiratory function of this organelle. Mitochondrial toxins such as azide and cyanide demonstrate inhibitory effects on virtually all respiring cells that utilize the cytochrome pathway (348). Agents
affecting conserved pathways would, presumably, target both the host and pathogen and would not be suitable targets for clinical use. Strategies developed to target the mitochondria of pathogens should benefit by focusing on differences or unique features between the host and the pathogen.

Ultrastructural studies on *C. albicans* cells treated with various imidazole antifungals (e.g., ketoconazole) have revealed pronounced morphological effects on mitochondria below *in vitro* inhibitory concentrations of 50 μg/mL (315,349). Imidazole antimycotics interact with the microsomal cytochrome P-450-dependent lanosterol 14α-demethylase system (260). Studies on isolated *C. albicans* mitochondria indicate that respiration inhibition occurs as a result of imidazole interaction with cytochrome oxidase (315). In addition to cytochrome oxidase, ketoconazole nonspecifically inhibits NADH oxidase and succinate oxidase at concentrations exceeding *in vivo* conditions (315). Another imidazole derivative, omoconazole nitrate, causes severe damage to mitochondrial cell membranes at *in vitro* fungicidal concentrations (255). *C. albicans* cells treated with a number of other antifungal agents such as riloprox, cicloprox olamine, and the pyrimidine analogs also show structural changes in organelles (49,112,280). However, *in vivo* effects of these antifungals on mitochondrial function have not been ascertained. Of the above antifungals, levels of only ketoconazole have been obtained in vivo. Studies with ketoconazole indicate that primary effects on mitochondrial function can be obtained at *in vivo* concentrations (349).

Several ubiquinone analogs cause direct inhibition of the mitochondrial electron transport and show broad spectrum antiparasitic activity (92,161). Atovaquone, a
hydroxynapthoquinone, disrupts the mitochondrial membrane transport chain in *Plasmodium yoelii*, through interactions with the cytochrome bc1 complex (106,330) and inhibits the synthesis of pyrimidines (144). Atovaquone is also effective against *Pneumocystis carinii* (87,164,165). Although controversial, *P. carinii* is considered by many to be a fungus (337,338). Atovaquone is currently in use as a therapeutic agent for AIDS-associated *P. carinii* pneumonia and has demonstrated extremely low toxicity in humans (87,165). The low toxicity may be due, in part, to the ability of humans to salvage preformed pyrimidines (87,144). Although there are no reports of the effects of atovaquone on other fungal pathogens, the potential for antimitochondrial therapeutic agents is encouraging. These data provide evidence that characterization of unique features of *C. albicans* mitochondria may lead to the uncovering of therapeutic targets.

**Investigation Background and Goals**

Cutler, et al., reported the presence of several putative high-molecular weight DNA repeat units in *C. albicans* (72). When total DNA was digested with *MspI* or *HpaII* (CCGG isoschizomers), a series of three fragments (4.2, 3.4, and 2.9 kb) were observed in ethidium bromide stained agarose gels. Based on the intensity of the bands, the fragments were postulated to be repetitive DNA. Furthermore, the presence of these fragments in ten separate *C. albicans* isolates, and the lack of similar fragments in other fungal and mammalian species, suggested that the putative repeats are species-specific. A 2.9 kb fragment was subsequently cloned from a *HpaII* digest of genomic DNA, and Southern blot hybridization analysis indicated that the DNA fragment was in fact, *C.*
*C. albicans* specific. The cloned DNA fragment was not the 2.9 kb putative repeat described above. Further investigation of the DNA clone revealed several interesting features (122,123). The DNA fragment, digested with *HpaII* and cloned into the *ClaI* site of pBR322, did not consist of a contiguous 2.9 kb fragment as expected. Restriction enzyme analysis revealed that three fragments from disparate regions of the *C. albicans* genome were ligated into the single *ClaI* site, generating a 5.5 kb insert. The 3.8, 1.6, and 0.79 kb fragments were extensively characterized (122).

The original intent of this thesis project was to clone and characterize the three high-molecular weight putative *MspI* repeats, as an extension of previous work (72,122,123). At the onset of this project, the origin of these putative repeats was unknown, although there was evidence that *MspI* fragments between 1.5 and 4.4 kb may represent mitochondrial DNA (123). I report here the cloning of several of the *MspI* repeats, and the mitochondrial origin of these fragments. A discussion of non-mitochondrial DNA repeats is given in Appendix I.

**Mitochondrial Genome Copy Number**

The reported difficulties in isolating petite mutants of *C. albicans* indicate that the mitochondrial genome is essential for viability (1,284). Although the mitochondrial genome has been characterized to a limited extent (284,309,367,368), important features such as the mitochondrial genome copy number is controversial. A previous report on the kinetic complexity of the repetitive fraction of *C. albicans* DNA, demonstrated that the major component (mitochondrial DNA) represented ca. 71% (367). Reassociation
coefficients were calculated corresponding to 74.8 kb repeated fragment with a reiteration frequency of 17 copies. Further analysis indicated that the mitochondrial genome was actually 40 kb, and not the 74.8 kb indicated by C_0t analysis (367), and the copy number was estimated at 30 per yeast cell (301). This number, however, is probably an overestimate because the inverted repeat regions, which account for almost one third of the mitochondrial genome, were not considered in the calculations (309,368).

The number of mitochondrial nucleoids, as evidenced by fluorescence microscopy, has been estimated to vary between 4.6 and 12.0 during the yeast cell cycle in *C. albicans* (171). Although the nucleoids were observed to be discrete particles and were assumed to contain a single genome, no attempts were made to quantify the number of genomes per mitochondrial nucleoid. Studies on *S. cerevisiae* indicated that the number of mitochondrial genomes per nucleoid varies between one and eight (371). No attempts were made to estimate the copy number of mitochondrial nucleoids in germinating *C. albicans* cells (13). The fluorescence studies relied upon visualization of DAPI-stained DNA, which does not discriminate between nuclear DNA and mitochondrial DNA. Although mitochondrial membranes were also stained with DAPSMI, there was no specific marker to differentiate between DNA types. The use of DNA probes to determine mitochondrial genome copy number per cell has been reported (278,352). Mitochondrial DNA probes a high degree of accuracy in such determinations (250,285). In my dissertation, I report on mitochondrial genome copy number during the yeast-to-hyphal transition as determined by use of mitochondrial
specific DNA probes. By using probes specific to either the mitochondrial genome or the nuclear genome, it was possible to determine copy number and origin of the DNA under study.

The localization of mitochondria to the hyphal apex of mature hyphae is believed to be an important event in fungal morphogenesis (372). The role of mitochondria early in the morphogenetic transition is less. As stated above, opposing results have been reported as to the respiratory activity of *C. albicans* in relation to cellular morphology. Land et al. proposed that dimorphism in *Candida* was the phenotypic result of a complex set of controls within the cell between cytoplasmic glycolysis and mitochondrial oxidative phosphorylation (205). Studies involving cofactors and inhibitors associated with electron transport showed that electron transfer away from flavoprotein is required for maintenance of yeast morphology, while conditions consistent with a buildup of reduced flavoprotein favored filament formation (206). Thus, filamentation in *C. albicans* is accompanied by repression of mitochondrial activity as evidenced by a change from an aerobic to a fermentative metabolism and from a high to a low respiratory activity. This indicates that filamentation requires less energy than yeast cell development. It is not clear whether the observed metabolic changes in *C. albicans* are related to the dynamics of organelle behavior during morphogenesis. Nor is it clear whether copy number changes in mitochondrial genomes accompany changes in mitochondrial activity. Levels of mitochondrial DNA in striated muscle cells (365) often accompany increased mitochondrial gene expression. In my dissertation work, I assayed two mitochondrial specific enzymes in order to determine if
changes in mitochondrial activity coincided with cell-cycle associated changes in mitochondrial DNA copy number. The specific activities of succinate dehydrogenase and cytochrome oxidase were determined to ascertain mitochondrial copy numbers at various time points in the yeast-to-hyphae transition. The results of these experiments are presented and their implications on morphogenesis are discussed.
Chapter 2

CANDIDA ALBICANS MITOCHONDRIAL GENOME COPY NUMBER AND MITOCHONDRIAL ACTIVITY DURING A YEAST TO HYPHAL TRANSITION

Introduction

Candidiasis often occurs as an opportunistic infection in immunocompromised individuals. Organisms in the Candida genus are the leading cause of life-threatening fungal disease and rank fourth among all bloodstream and urinary tract nosocomial infections in the USA (174). The prevalent cause of candidiasis is C. albicans, which normally resides as a commensal on human mucosal surfaces. This species is polymorphic, as it can exist in either yeast or filamentous forms. The ability of C. albicans yeasts to undergo a hyphal transition has been speculated to be a virulence trait (111,216). Although morphogenesis has been extensively studied in C. albicans, little is known about the role of mitochondria (mt) for a yeast to hyphal transition.

Unfortunately, there are a limited number of cost-effective and safe antifungal agents for treatment of systemic candidal infections. As a result, there has been a major expansion in antifungal drug research to identify and characterize new targets (141). The main focus of our work was to assess mt changes during a yeast to hyphal morphogenesis in C. albicans. Thus, these studies may also yield new insights into potential drug targets.
through an understanding of the role of mt in the morphogenic transition of *C. albicans*. Hydroxynaphthoquinone therapeutic agents directed against the mt of other human pathogens are effective (164), and substantiate the rationale behind mt research.

Mt are highly variable organelles in size, shape, chemical composition and function (298). They serve primarily as a site of oxidative phosphorylation in eukaryotic cells. In addition, mt also synthesize heme, lipids, amino acids, and mediate the intracellular homeostasis of inorganic ions (348). The proper function of this organelle is essential in humans, as evidenced by recent discoveries that certain diseases may be caused by mt anomalies (218). The importance of mt in fungi is less clear. Studies on petite mutants in *Saccharomyces cerevisiae* indicate that function of an intact mt genome is required for the development of a respiration-competent organelle (16), but *S. cerevisiae* can survive without mt function, albeit with a prolonged generation time (103). Unlike *S. cerevisiae*, there have been no reports of viable mutations in the *Candida albicans* mt genome, which indicates that the genome of this organelle is essential for *C. albicans* growth.

The *C. albicans* mt genome is a double stranded DNA circle of 40 kb (367), and contains a large inverted repeat that can undergo recombination (309). Although a few characteristics of the mt genome have been defined, basic information such as mt genome copy number is either lacking or is controversial. Reassociation kinetics of *C. albicans* repetetive DNA, demonstrated that the major component (mtDNA) represents ca. 71% (367). The reassociation coefficient corresponded to ca. 74.8 kb and the reiteration frequency was extrapolated to be 17 (mt genome copies). Both of these values were
apparently incorrect. Based on the actual size of the mt genome (40 kb) as determined by Wills et al., (367) and the data from mini-C_{0}t analysis, the copy number was re-estimated at ca 30 (301). Others reported variations between 4.6 and 12.0 per cell in mt nucleoid numbers during \textit{C. albicans} yeast cell cycle events (171). Unfortunately, their approach relied upon visualization of fluorescently labeled DNA and did not differentiate mt genomes from mt nucleoids.

Mt genome copy numbers in human cells have been estimated by use of DNA probes (278,352). This approach reportedly yields numbers that are accurate to within \pm 10\% (250,285). We used the mt specific probe strategy to directly address variations in genome copy number during a yeast-to-hyphal transition in both wild-type \textit{C. albicans} strains and null hyphal mutant strains. Signals obtained from hybridization analysis of the mt probes were compared to signals from the single copy actin gene (226) to calculate copy number. In addition, mt enzyme activities were determined during the transition to assess whether mt genomic copy number is coupled to mt organelle number. Based on previous findings that \textit{C. albicans} mt undergo a series of fusion and fragmentation events during the yeast to hyphal transition (13,343), we assayed mt marker enzymes in both cell homogenates and enriched mt fractions to determine if enzymatic dynamics are coupled to changes in mt genomic copy number. Our data indicate that mt functional changes precede mt genome and organellar replication during a yeast to hyphal transition, and imply that mt play an important role in a yeast to hyphal transition.
Materials and Methods

Strains, Media, and Culture Conditions

*C. albicans* strains Ca1 (145), 9938 (146), and 3153A (ATCC 28367) are clinical isolates. CA2 is an echinocandin resistant, agerminative mutant of strain 3153A (57). Strains CA2 and 3153A were kindly provided by A. Cassone (Instituto di Microbiologia, University of Rome, Italy). All other strains of *C. albicans* are from the MSU stock collection. *C. albicans* yeast cells were grown from frozen glycerol stocks as previously described (181) and the epithet for each was confirmed by API 20C testing (Analytab Products, Inc. Plainville, N.Y.). *C. albicans* yeasts were obtained by growth at 37°C under constant aeration by rotation of culture flasks containing the growth medium, glucose-yeast extract-peptone broth (GYEP). *C. albicans* germ tubes were serum-induced. Yeast forms of *C. albicans* were grown on Sabouraud dextrose agar at 37°C for 48 h, harvested, washed in deionized water and transferred to RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10mM HEPES (Sigma) and 5% normal calf serum (HyClone Laboratories, Logan, UT) at a concentration of 2 x 10^6 cells/mL. Germ tube development occurred by incubation at 37°C under aeration by rotary shaking at 160 rpm. Germination under these conditions was >90%. Incubation times varied in duration and are specified in the figure legends. *E. coli* SURE cells [e14^*(McrA*) D(mcrCB hsdSMR mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC [F' proAB lacI97 Z D(M15 Tn10] (Stratagene, LaJolla, CA) were used for transformation and propagation of plasmids and were grown according to the manufacturers directions. pCaActI containing the *C. albicans* actin gene was described by Mason et al. (226) and was a generous gift from W.S. Riggsby (University of Tennessee, Knoxville, Tennessee).
**Pulsed Field Gel Electrophoresis**

Total *C. albicans* DNA was prepared as plugs in 1% SeaPlaque LMP agarose (FMC Rockland, ME) according to previously described methods (23). Intact *C. albicans* mt fractions were similarly prepared by suspension in 1% LMP agarose and lysed in 100 mM EDTA, 1% lauryl sarcosine, pH 8 at 37°C for 2 h. Chromosomes and intact mt DNA were separated by CHEF analysis on a Gene Mapper (BioRad, Richmond, CA) and molecular sizes were estimated by use of *Saccharomyces cerevisiae* commercial size standards (BioRad). Gels were cast with 1% PFGE grade agarose (BioRad) in 0.5X TBE. All gels were run at 14°C in 0.5X TBE at 6 V/cm and using a 120° rotation angle. Pulse time was ramped from 50 to 90 seconds over the relatively short time of 16 h to prevent the 40 kb mt genome from eluting from the gel.

**Isolation of DNA**

*C. albicans* mtDNA was purified from enriched mt fractions. Mt fractions were obtained by mechanical disruption of spheroplasts and sucrose density gradients as described by Wills et al. (367). *C. albicans* total DNA was isolated from both yeasts and hyphae that were lysed upon exit from a cold pressure cell (Refrigerated Ribi-Sorvall cell fractionator model RF-1) according to the method of Glee et al. (124), with the following modifications. In all steps, cells and solutions were maintained near 0°C in an ice/water slurry until lysed. Hyphal cells were harvested by filtration through a 1.2 μm porosity mixed cellulose ester (type RA) filter (Millipore, Medford, MA), washed in sterile distilled water, and suspended in breakage buffer at a concentration of 2 x 10⁹ cells/mL. The suspension of cells was placed in the Ribi pressure cell which had been pre-cooled to 4°C. The cell suspension was immediately passed once through at 45,000 psi and the effluent containing broken fungal cells was collected in a vessel submerged in
ice. The efficiency of breakage was >90% as determined by microscopic examination. Separation of DNA from other cellular constituents was done as described previously (124).

**DNA Cloning**

Standard methods were employed for DNA manipulations (15). *MspI*-digested mtDNA was ligated into the *Clal* site of pBluescript II S/K+ (Stratagene) by use of a DNA ligation kit according to the manufacturer's instructions (Stratagene). Electrocompetent *E. coli* SURE cells (Stratagene) were prepared according to previously described methods (89) and transformed using an Electroporator II unit (Invitrogen, Carlsbad, CA) set at 50 μF capacitance, 150 ohm resistance and 1500 volts. Cells were allowed to recover by incubation at 37°C for 30 min in LB medium and plated on LB agar containing ampicillin. Transformants were chosen by blue/white color selection using methods specified by Ausubel et al. (15). Insert DNA was excised with *MspI* and analyzed by Southern blot hybridization (329) as indicated below.

**Hybridization Analysis**

Restriction fragments, chromosomes and mtDNA were separated by electrophoresis in agarose gels and depurinated by treatment with 0.25 N HCl for 15 min at 22-24°C (R.T.). DNA was denatured in 0.4 N NaOH for 15 min at R.T. and transferred overnight onto GeneScreen Plus membranes (DuPont/NEN, Boston, MA) by capillary action (329). *C. albicans* actin and mtDNA inserts were purified from vector DNA by gel purification with Gelase according to the manufacturers directions (Epicenter Technologies, Madison, WI) or by phenol extraction (15). Probes were labeled with either [α-32P]-dCTP (DuPont/NEN) by nick translation (15) or [γ32P]-ATP by use of a
5'-end labeling kit (Boehringer Mannheim, Indianapolis, IN) per the manufacturers instructions. All labeling reactions were purified on Elutip-D columns (Schleicher & Schuell, Keene, NH). After purification, the specific activities of radiolabeled probes were measured in a liquid scintillation counter. Membranes were prehybridized for 4 h at 45°C in a hybridization solution containing 6X standard saline citrate (1X SSC, 150 mM NaCl, 15 mM sodium citrate), 50% deionized formamide (Ambion, Austin, TX), 2X Denhardt's solution (1X, 1% (v/v) Ficoll 400, 1% (v/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin) and 50 mM sodium phosphate, pH 7.0. 32P-labeled probe was denatured (15 min, 95°C) and added to give 2 x 10^6 cpm/ml. Hybridization was for 16 h at 45°C in a hybridization oven (Bellco Glass Co., Vineland, N.J.). Filters were washed 3 times in 2X SSC, 0.1% (w/v) sodium dodecyl sulfate (SDS) for 15 min at RT and then 2 times in 0.1% SSC, 0.1% SDS for 30 min at 50°C. After air drying, membranes were analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Dot Blot Analysis

*C. albicans* total DNA was extracted and purified from Ca1, 3153A, 9938, and CA2 at times 0, 30, 90 and 180 min of transfer into hyphae-inducing medium containing serum. These samples were digested with *Hind*III and denatured by boiling for 10 min in 0.4 N NaOH. After cooling in an ice water slurry the DNA was dot blotted onto GeneScreen Plus membranes (DuPont/NEN) in a Bio-Dot microfiltration manifold (BioRad). Six replicates were prepared for each DNA sample. Single-stranded DNA was blotted directly onto the membranes in 0.4 N NaOH. The positively-charged nylon blots were hybridized and washed as described for Southern blot analysis (329). Dot blot signals were quantified by use of the Imagequant software package (Molecular Dynamics). Total counts obtained by hybridization with mt specific probes were
compared to counts generated from identical blots probed with the _C. albicans_ single copy actin gene (per haploid genome). Because _C. albicans_ is diploid, the mt genome to actin ratio was divided by two to account for the two copies of the actin gene per diploid genome. To normalize DNA loading, DNA samples were adjusted to similar concentrations based on optical density at 260 nm. DNA samples were then serially diluted in two-fold increments, dot-blotted onto GeneScreen Plus membranes, and probed with 0.5 μg of 32p-end labeled _C. albicans_ actin probe. Hybridization was quantified to determine the linear range for both the GeneScreen Plus membranes and the phosphorimager. The necessity to correct for cytidine content was circumvented by the use of 5' end-labeling (225).

Preparation of _C. albicans_ Homogenates and Mt for Enzyme Assays

_C. albicans_ yeast and hyphal cells were washed three times with cold phosphate-buffered saline and pelleted by centrifugation at 5,000 x g at 4°C. Cells were suspended in a volume of homogenization solution (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5) equal to 10 times the volume of packed cells and broken by glass beads as previously described (147). Unbroken cells were removed by centrifugation at 1,500 x g. Aliquots of the homogenate were set aside for determinations of protein concentration by use of a microtiter plate BCA protein assay kit (Pierce, Rockford, IL) per manufacturer's recommendations. Bovine serum albumin (BSA) was supplied in the kit and was used as the protein standard. The remainder of the homogenate was used either directly for enzyme activity determinations or for the isolation of a mt fraction as follows. The homogenate was diluted 1:1 (v/v) with homogenization solution and centrifuged at 1,500 x g for 10 min at 4°C. The supernatant solution was removed and centrifuged at 20,000 x g for 20 min to obtain a crude mt pellet. The pellet was suspended in the original volume
of homogenization solution and the protein concentration determined by BCA protein assay (Pierce). Cellular homogenates and crude mt fractions were stored at -80°C until use in the enzyme assays described below.

**Succinate Dehydrogenase Activity**

The *C. albicans* strains, culture conditions, and time points were identical to those used in the assessment of mt genome copy number. Enzyme specific activities were determined for both cellular homogenates and membrane enriched fractions (crude mt). Colorimetric assays and an automated microtiter plate reader were employed to accommodate the large numbers of samples and to allow for multiple time readings. Activity of *C. albicans* succinate dehydrogenase was measured by a modification of the phenazine methosulfate assay as previously described (224). All enzyme assays were performed in 96-well microtiter assay plates (25880-96; Corning, Cambridge, MA) and each sample was done in triplicate. The reaction mixture contained 50 mM potassium phosphate, pH 7.4, 2 mM sodium cyanide, 25 mM sodium succinate, 1 mM phenazine methosulfate (PMS), and 0.1 mM dichloroindolphenol (DCIP) in a final volume of 200 µL. The final protein concentrations in the assays were 3.5-4.5 µg/mL for homogenates and 1.5-2.5 µg/mL for mt fractions. After a 3 min preincubation at 30°C, reactions were started immediately by use of a multichannel pipettor for addition of phenazine methosulfate and DCIP. The linear decrease in absorbance at 600 nm due to the oxidation of DCIP was measured by use of a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA). Plate readings were taken at 15 sec intervals over a 30 min period. *Km* values were directly calculated using the SOFTmax PRO software package (Molecular Devices). Negative control wells contained all components except succinate.
One unit of enzyme activity equaled 1 nmol of DCIP oxidized per minute at 30°C, as calculated by use of the DCIP millimolar extinction coefficient of 19.1.

Cytochrome C Oxidase Activity

The oxidation of cytochrome c was measured by observing the 550 nm optical density change of the solution with time as described by Wharton and Tzagoloff (361), but with the following modifications. The cytochrome c oxidase assay was adapted to a microtiter format similar to the above succinate dehydrogenase assay. The reaction mixture contained 12 mM K2HPO4-KH2PO4, pH 7.0 and 140 μg of reduced cytochrome c. A 10 mg/mL solution of horse heart cytochrome c (C-7752; Sigma) was fully reduced by titration with a 100 mM solution of sodium dithionite until maximum absorbance at 550 nm was achieved. In the assay, 1.0-1.5 μg of protein was used for cellular homogenates and 0.5-1.0 μg for the mt preparations. The reaction was started by the addition of reduced cytochrome c and readings were taken at 15 second intervals over a period of 40 min at 30°C. Calculation of enzyme kinetics was similar to the succinate dehydrogenase assay by use of a millimolar extinction coefficient of 18.5 for cytochrome c.

Results

Distribution of MspI Digested *C. albicans* MtDNA

*C. albicans* mtDNA, and total DNA (nuclear and mtDNA) from various strains of *C. albicans* were completely digested with *MspI* and electrophoresed through an agarose gel (Fig. 2, Panel A). A similar restriction pattern was observed between the different *C. albicans* strains (e.g., observe bands at 4.1, 3.6, and 3.4 kb). An additional intense-staining band was also observed in lanes 6-8 at about 4.8 kb, which we presumed was due
Figure 2. Moderately sized putative repeats are mt in origin. (A.) *MspI* restriction fragment pattern of DNA from various strains of *C. albicans* and mtDNA. Lane 2 contains mtDNA from *C. albicans* strain Ca1. Lanes 3 to 8 contain total DNA from *C. albicans* Ca1, 3153A, A9, 105, 9938, and 222a, respectively. Each DNA sample was digested with *MspI*, electrophoresed in 1% agarose, and stained with ethidium bromide. Lane 1 contains lambda DNA digested with *HindIII* as a size standard. The position of three of the putative repeats are noted (e.g. 4.1, 3.6, 3.4 kb) (B) Autoradiogram of gel from panel A that was blotted onto a nylon membrane and probed with $^{32}$P nick-translated mitochondrial genomic DNA.
to *MspI*-RFLP differences among *Candida* strains. *MspI*-digested mtDNA from strain Cal yielded fragments at $\leq 4.1$ kb, which corresponded to the intense bands in the total DNA digests (Fig. 2, Panel A, lane 2), with the exception of the higher molecular weight bands in lanes 6-8.

To confirm that mtDNA was represented by the intense ethidium bromide bands in lanes containing *MspI* restriction fragments from total cellular DNA, the gel in Fig. 2, Panel A was blotted onto a nylon membrane and probed with nick translated mtDNA (Fig 2, Panel B). Four of the *C. albicans* strains had essentially identical, mt RFLP patterns (lanes 3-6). There were, however, a few significant differences in restriction site polymorphisms noted for strains 9938 and 222a as indicated by lanes 7 and 8, respectively. The additional band of 4.8 kb in lane 6 did not hybridize with mtDNA. Regardless of *MspI* RFLPs among *C. albicans* strains, mtDNA isolated from a single strain cross-hybridized with all strains and, thus, can be used as a probe for the determination of mtDNA copy number.

**Cloning of MtDNA**

*C. albicans* mtDNA was digested with either *MspI* or *KpnI*. *MspI*-generated fragments were cloned by ligation into the *ClaI* site of pBluescript II (pBSII), which resulted in the loss of the *ClaI* recognition sequence. The vector pBSII contains several *MspI* sites, including sites directly upstream and downstream of the original *ClaI* site. The plasmid clones were digested with *MspI* to remove the majority of the vector sequence. The resulting mtDNA fragments are flanked by 27 bases of pBSII on the 5' end and 30 bases on the 3' end, and the remaining vector fragments were smaller than 500 bases. An array of different sized plasmids were generated. Hybridization analysis of the ligation reaction revealed the presence of all mtdna *MspI*-generated fragments except for
the 4.1 kb band (data not shown). *MspI* mt clones (lanes 3-5) and mtDNA were digested with *MspI* (Fig. 3). The inserts from *C. albicans* mt clones Ib10 (Lane 3), Ib13 (Lane 4), and Ib39 (Lane 5) were purified and used for subsequent investigations.

Digestion of the mt genome with *KpnI* yielded five fragments ranging in size from 13 kb to 1.6 kb (Fig. 3, Lane 6). The background smear was likely degraded mtDNA and not contaminating nuclear DNA, as evidenced by the lack of a hybridization signal when *C. albicans* actin DNA was used as a probe (data not shown). Furthermore, undigested mtDNA showed the background smear (data not shown). The two largest fragments (13 kb and 9.1 kb) each contain one complete arm of the inverted repeat (368). We cloned several of the *KpnI* fragments into pBSII, including the 9.1 kb DNA (clone K2) containing one of the inverted repeats (Lane 7). The mtDNA insert in K2 contains

![Figure 3. *MspI* and *KpnI* C. albicans mt DNA clones. Lane 2 contains C. albicans mtDNA digested with *MspI*. Lanes 3 to 5 contain *MspI* digested mtDNA clones Ib10, Ib13, and Ib39, respectively. Lane 6 is mtDNA digested with *KpnI*. Lane 7 contains the *KpnI* digested mitochondrial clone K1. The 9.1 kb fragment containing the inverted repeat is labeled in the margin. Lane 8 is clone K1 digested with *PvuII*. Lane 1 is a 1 kb molecular weight ladder.](image-url)
two internal PvuII restriction sites, and pBSII contains two PvuII sites. PvuII digestion of K2 resulted in four fragments (Lane 8) as expected. Three of the fragments contained portions of pBSII (2.5, 3.7, 4.8 kb). The fourth fragment is a 1.2 kb mt fragment and contained no vector DNA. This inverted repeat fragment was one of the probes used in the experiments in table 3.

Specificity of Mt Probes

To determine the specificity of the 2.9 kb fragment (1b13) to the mt compartment, a Southern-blot analysis was performed using mtDNA and total DNA digested with a series of restriction enzymes. *C. albicans* DNA was cut with *MspI*, *EcoRI*, *HindIII* and *BamHI* and electrophoresed in an agarose gel (Figure 4, Panel A). Blots were probed with 1b13. 1b13 was conserved in RFLP analysis of different *C. albicans* strains and was chosen as a representative fragment for a single mt genome for use in copy number determination. In each restriction digest, mtDNA probe 1b13 hybridized to the same sized fragments in both the mtDNA lanes and the total DNA lanes (Fig. 4, Panel B), which shows that the probe does not cross-hybridize with nuclear fragments of different size. Identical results were obtained with similar blots probed with the 1.4 kb (1b13) and 1.7 kb (1b39) *MspI* mt fragments (data not shown).

Specificity of the mt probe 1b13 to the mt compartment was further demonstrated using Southern-blot analysis of pulsed-field gels. The considerable size difference between most yeast mt and nuclear genomes facilitates separation with pulsed-field electrophoretic techniques (321). We modified the chef gel conditions to obtain optimal resolution of the 40 kb candidal mt genome (Fig. 5, Panel A). Fractionated *C. albicans* mtDNA was included as a control (Fig. 5, Panel A, Lanes 4-5). Fig. 5, Panel B shows a Southern-blot of the CHEF gel probed with the nick-translated mtDNA clone, 1b13. This
Figure 4. (A.) Restriction digest analysis of *C. albicans* total DNA and mtDNA. Each restriction enzyme was used to cut one sample of total DNA and one sample of mtDNA. Lanes 2 contains *MspI* digested mtDNA. Lane 3 is *MspI* digest total DNA. Lanes 4 and 5 contain *EcoRI* digested DNA. Lanes 6 and 7 contain *HindIII* digested DNA. Lanes 8 and 9 are digested with *BamHI*. Lane 1 is a lambda *HindIII* digest as a molecular weight marker. (B) Autoradiogram of gel from panel A, which was blotted and probed with mt clone 1b13.
clone contains regions of both the cytochrome b gene and cytochrome oxidase subunit I as evidenced by sequence analysis (GenBank AF080074). When clone 1b13 was used as a probe, it hybridized to the 40 kb mt band in both the mt controls (Lanes 4-5) and the

Figure 5. Mt probe 1b13 is specific to the mt compartment. (A.) Pulsed field electrophoresis of *C. albicans* total and intact mtDNA. Agarose plugs containing either *in situ* lysed *C. albicans* cells, or intact mitochondria were run on a 1% agarose gel under conditions specified in the methods section. Lanes 2 and 3 contain *C. albicans* total DNA in increasing concentration. Lanes 4 and 5 contain mtDNA. The position of the 40 kb mitochondrial genome is noted in the margin. (B) Southern blot analysis of gel from panel A. Blots were probed with $^{32}$P nick-translated 1b13 insert DNA. (C) Autoradiogram of a similar blot probed with $^{32}$P labeled *C. albicans* actin DNA.
total *C. albicans* lanes (2-3), but not to the chromosomal regions. Fig. 5, Panel C is a Southern-blot of a similar gel probed with nick-translated *C. albicans* actin DNA. The actin probe hybridized to the chromosomal region, but not to mtDNA. Comparable results were obtained on similar gels probed with the entire mt genome (data not shown). From these results we concluded that the mtDNA probes did not cross react with *C. albicans* nuclear DNA. Due to mt specificity, we determined that the probes could be used for the analysis of mt genome copy number in blots of *C. albicans* total DNA.

Mt Genome Copy Number as Determined by Hybridization Analysis

Copy number changes of *C. albicans* mt genomes were assessed during a yeast-to-hyphal transition. A series of dot-blot analyses were employed to quantify and compare mt genome numbers in strains Ca1, 3153A, 9938, and CA2. Ca1 and 3153A produced normal hyphae under the germination conditions, but 9938 produced only pseudohyphae and CA2 grew as yeast cells only. Increases in mtDNA copy numbers were observed between times 0 and 90 min in strain Ca1, and a steady state was achieved between times 90 and 180 min (Table 3). There were approximately 3.4 mt genomes in stationary yeast cells as compared to 4.8 copies in 3 hr germ tubes. The inverted repeat probe, K1, showed values approximately twice the value of 1b13 (6.6 for yeasts and 9.5 for germ tubes). Strains 3153A, 9938, and CA2 also showed an increase in mt genomes between times 0 and 180. However, the relative copy number at time 180 in the non-germinating strain, CA2 and the pseudohyphal producing strain, 9938, were higher than those achieved by the normal strains Ca1 and 3153A. Copy numbers at 180 min for CA2 and 9938 were 6.3 and 5.0, respectively. Similar results were obtained using mt probes 1b10 and 1b39 (data not shown). The mt genome copy number differences between yeast and hyphal cells indicated a possible change in mt respiratory activity and/or a
change in the number of mt organelles. To address this issue, we assayed the respiratory activity of two mt marker enzymes during a yeast to hyphal transition.

Table 3. Mitochondrial genome copy numbers in germinating *C. albicans* cells obtained from mitochondrial-specific DNA probes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Timea</th>
<th>32P 5' End-labeled mitochondrial probes</th>
<th>1B13b</th>
<th>K1b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca1</td>
<td>0 Min</td>
<td>3.43 ± 0.09</td>
<td>6.58 ± 0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30 Min</td>
<td>3.75 ± 0.14</td>
<td>7.94 ± 0.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90 Min</td>
<td>4.78 ± 0.19</td>
<td>9.62 ± 0.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>180 Min</td>
<td>4.77 ± 0.13</td>
<td>9.54 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>3153A</td>
<td>0 Min</td>
<td>3.66 ± 0.21</td>
<td>6.02 ± 0.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>180 Min</td>
<td>4.51 ± 0.17</td>
<td>9.25 ± 0.47</td>
<td></td>
</tr>
<tr>
<td>9938</td>
<td>0 Min</td>
<td>3.27 ± 0.16</td>
<td>6.12 ± 0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>180 Min</td>
<td>5.01 ± 0.19</td>
<td>10.79 ± 0.32</td>
<td></td>
</tr>
<tr>
<td>CA2</td>
<td>0 Min</td>
<td>3.23 ± 0.11</td>
<td>6.29 ± 0.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>180 Min</td>
<td>6.31 ± 0.21</td>
<td>12.44 ± 0.54</td>
<td></td>
</tr>
</tbody>
</table>

a Expressed as length of time after incubation in hyphal-inducing medium.

b Data are expressed as copy number ± the standard deviation of 6 replicates. All data are normalized against signals obtained from similar blots probed with the *C. albicans* single copy actin gene.

Analysis of Mt Enzyme Activities During Morphogenesis

Cytochrome oxidase and succinate dehydrogenase specific activities were determined for *C. albicans* cellular homogenates and mt in cells undergoing a yeast to hyphal transition (Fig. 6). For strain Ca1, succinate dehydrogenase activity in crude mt preparations declined rapidly during the early stages of germination, as compared to the specific activity in the cellular homogenates which slightly increased (Fig. 6, panel A).
Figure 6. *C. albicans* succinate dehydrogenase and cytochrome oxidase specific activity changes during a yeast to hyphal transition. For each assay, the specific activities of crude mt fractions (squares) and cellular homogenates (circles) were measured at times 0, 30, 90, and 180 min of germination. Specific activities are expressed as nmoles substrate reduced/min/mg protein. Samples were performed in triplicates and bars representing standard deviation are represented on the graphs. (A). Succinate dehydrogenase assay of *C. albicans* strain Ca1 mt fractions and cellular homogenates. (B). Cytochrome oxidase assay of the same samples in panel A.

Conversely, the specific activity of cytochrome oxidase increased for the same mt preparation (Fig. 6, panel B). Although showing opposite trends in activity especially during the first 30 min of germination, changes in both succinate dehydrogenase and cytochrome oxidase leveled off between 90 and 180 min as indicated by a change in slope between these time points. These results indicate that *C. albicans* mt undergo specific changes in enzyme composition early in the morphogenetic transition.
Discussion

Cutler et al. (72) previously reported on the presence of several high-molecular DNA repeat units in *C. albicans*. When total DNA was digested with *MspI*, three fragments (4.2, 3.4, and 2.9 kb) were observed in ethidium bromide stained agarose gels. Based on the intensity of the bands, the fragments were postulated to be repetitive DNA. Furthermore, the presence of these fragments in ten separate *C. albicans* clinical isolates, and the lack of similar fragments in other fungal and mammalian species, suggested that the putative repeats are species-specific. In this report, we show that the high molecular repeats are mt in origin. Digestion of *C. albicans* total and mtDNA with *MspI* yielded similar restriction fragment patterns (Fig. 2). The mtDNA probed to the position of the putative repeat units in the total DNA cut and the mtDNA probes hybridized only to the position of the mitochondrial band in a pulse-field gel electrophoretic separation of nuclear and mitochondrial chromosomes (Fig. 5). From these results, we concluded that the conserved repeat elements generated as a result of *MspI* digests of total cellular DNA is due to the mt genome, and not due to genomic fragments of similar molecular weights.

The conserved nature of mt DNA restriction patterns is in direct agreement with previous studies (102,263). The suggestion that *Candida* mtDNA contains species-specific sequences (102) was proven by Miyakawa et al. (244). In the latter study, the species-specific fragment was located in the inverted repeat region of the mt genome and hybridized to clones E2 and E3 of a mt EcoRI library (367). The three high molecular weight mt *MspI* fragments (4.2, 3.4 and 2.9 kb) described here hybridized to clones E4, E5, and E6 of the *EcoRI* library (data not shown).
The primary focus of this study was to determine mt genome copy numbers during the yeast to hyphal transition in *C. albicans*. Previously, mt copy number determination in germinating *C. albicans* cells relied on either electron (343) or fluorescence microscopy (13) to visualize and count individual mt. Both of these groups reported a mt fusion event at the initiation of germ tube formation, and subsequent fragmentation during germ tube elongation. Efforts were not made to determine the mt genome copy number by the electron microscopic approach. In the fluorescence studies, the number of mt nucleoids was estimated at 8 copies per mature blastoconidium (171), however, no attempts were made to estimate the copy number in germinating cells (13). This approach relied upon visualization of 4’, 6-diamidino-2-phenylindole-stained DNA, which does not discriminate between nuclear DNA and mtDNA. Mt membranes were also stained with 2-(4-dimethylaminostyryl)-1-methylpyridinium idodide, but there was no specific marker to differentiate between DNA types.

Alternatively, DNA hybridization techniques provide both specificity and accuracy (± 10%) in the determination of mt genome copy number (250). For our study, we chose a mt probe that did not cross react with nuclear DNA, as evidenced by both restriction enzyme and pulsed field analysis (Figs. 4,5). The specificity of lb13 to the mt compartment and the actin gene to the nucleus, allowed mt genome copy number determinations to be made directly on *C. albicans* total DNA samples. By this approach, we computed that stationary phase yeast cells have 3.5 mt genome copies per yeast cell, regardless of the strain (Table 3). In serum-containing medium, increases in copy number were observed through 90 min of incubation and remained constant to 180 min. Differences were observed between wild-type and mutant strains at 180 min of incubation. Wild-type strains averaged 4.77 and 4.51 for Cal and 3153A, respectively. Strain 9938, which produces only pseudohyphae in serum, yielded 5 copies per cell and
the agerminative mutant CA2 showed 6.3 copies. Our results suggest that the replication of mt genomes is associated with changes in both cellular morphology and growth. An increase in the overall number of mt genomes in strain CA2 may be indicative of increased respiratory demands of actively growing yeast cells, as compared to either stationary phase yeasts or young hyphae (e.g., 3 hr germ tubes). Stationary phase yeast cells would be expected to have a relatively low requirement for mitochondrial function because replication and macromolecular synthesis is considerably less than that of yeast cells in log phase growth (259). The intermediate number of mt genomes in 9938 may be the result of their ability to form pseudohyphae, but not true germ tubes. Our results suggest that there is increased respiratory demands for development of elongated structures, but pseudohyphae require less than hyphae. Increased respiratory demands of young hyphae is consistent with finding that mt are required for hyphal induction (206).

*C. albicans* yeast cells treated with the mt-inhibiting dyes, acridine orange, ethidium bromide and quinacrine led to repression of filamentation in a germination-inducing medium. An alternative explanation for increased copy numbers in the hyphal deficient mutants CA2 and 9938 is that a mutational event affected regulation of mt replication. Both the wild-type parent strain (3153A) and its spontaneous mutant (CA2), however, showed similar mt genome copy numbers in stationary yeast cells, which indicates that the changes were morphotype-specific.

The values we obtained for mt genome copy number in *C. albicans* yeast cells is considerably lower than previously reported estimates and there have been no reports of mt copy number in germinating cells. In a review of *C. albicans* genetics, Scherer and Magee (301) estimated 30 mt genome copies per yeast cell based on data from C_0t analysis (367). The calculation of this figure is unclear because the data from the original C_0t report indicated a 74.8 kb mt component with a reiteration frequency of 17 (i.e., 17
copies of a 74.8 kb mt genome). Although Wills et al. (367) determined the actual size of the mt genome to be 40 kb, and not 74.8 kb, the copy number discrepancy between 17 and 30 was not addressed.

One possible explanation for our lower values, is that actin may be present in multiple copies in the *C. albicans* genome. This is unlikely, however, because others have shown actin as a single copy gene per *C. albicans* haploid genome (226). Another possibility is that the final mt genome copy numbers were skewed because of differential hybridizations of the actin and 1b13 mt probe. The skewing could be due to either differences in probe length, the 1b13 is 2.9 kb and the actin is 2.0 kb, or because of differences in percent G+C ratios. We addressed the influence of differential probe lengths by inclusion of the mt *MspI* probes 1B10 (1.4kb) and 1b39 (1.9kb) in the dot blot analysis. Copy number determinations were essentially identical for all three mt genome probes (data not shown). To address the possible effect of percent G+C differences between the actin probe and mt probe 1b13, mt genome copy number was obtained by use of two kinds of labeled probes; nick translated and 5′ end-labeled. 1b13 to actin ratios were virtually identical in both cases. As a further confirmation of our results, the use of the inverted repeat probe K1 gave twice the signal as 1B13. The possibility that the number of mt genomes per fungal cell is a function of strain variability also seems unlikely because we obtained essentially the same results for the two wild-type *C. albicans* strains, Cal and 3153A.

Based on previous findings that *C. albicans* mt undergo a series of fusion and fragmentation events during the morphogenetic transition (13,343), a second aim of this study was to determine whether mt function changes in response to hyphal production. In several fungal species (e.g. *Hansenula wingei* and *Schizosaccharomyces pombe*), SDH is entirely encoded in nuclear genes, whereas the mt genomes encode three subunits of
COX (267,308). The mt genome of *C. albicans* also contains three subunits of COX (284). Although the specific location of SDH in the *C. albicans* genome is unknown, sequence analysis of 3 SDH subunits indicate that they are nuclear in origin (http://alces.med.umn.edu). In our studies, both SDH and COX assays yielded different findings in *C. albicans* cells undergoing a yeast to hyphal transition. SDH specific activity in crude mt was characterized by a rapid decrease between 0 and 90 min of germination, whereas COX showed a considerable increase in specific activity during these same time points as compared to only a slight increase in cellular homogenates (Fig. 6). These findings indicate that between 0 and 90 min of germination, respiratory changes in *C. albicans* are related to enzyme concentrations of individual mt, and not to an increased number of organelles. However, specific activities of crude mt preparations in both SDH and COX leveled off between times 90 and 180 min and approached a similar slope for specific activity changes in the cellular homogenates. This indicates that at 90 min the number of mt or mt volume per cell begins to increase and that there are fewer changes in the SDH and COX levels in individual mt. As mentioned previously, the mt genome copy number also increased between 0 and 90 min of germination and leveled off between 90 and 180 min. Together, the data from mt genome copy number analysis and enzyme specific activity determination indicate that increases in mt genome copy number are not necessarily coupled to increased numbers of mt organelles. Also implied in this study is that the mt genome replication occurs prior to 90 min of germination and is followed by mt organelle replication or growth between 90 and 180 min. Concurrent with the increased number of mt genomes early in morphogenesis was a decrease in respiratory activity as indicated by the change in SDH activity. This finding is consistent with the sustained decrease in O₂ consumption in 120 min *C. albicans* germlings shown by Land et al. (206) and Yamaguchi (375). The higher levels of COX
activity do not necessarily reflect increased initial respiration in developing germ tubes. Although the respiratory activity of hyphae is controversial (10), our data indicate that hyphae do not show an increased capacity for oxidative phosphorylation prior to complete expression of tricarboxylic acid cycle intermediates e.g., succinate dehydrogenase. Our studies did not directly address the fragmentation of mt organelles during the initiation of germination reported by others (13,343). The specific activity determinations we employed would not have detected mt fragmentation events unless accompanied by a change in total organelle volume. Tanaka et al. (343) reported that organelle volume to total cell volume remained constant during fragmentation events. Further studies are required to determine whether individual mt organelles contain a mt genome during the fragmentation process.

Based on the current understanding of *C. albicans* mt, it is tempting to speculate that both fragmentation and mt enzyme changes fulfill temporary energy requirements for the initiation of germination and that these individual mt fragments do not require a mt genome. Coalescence of these fragments prior to cell division would allow the appropriate segregation of a mt genome to both parent and daughter cells. Further investigations are necessary to understand these processes in *C. albicans* growth and development. The apparent lack of mt petite mutants in *C. albicans* indicates that faithful replication and segregation of the mt genome is required for *C. albicans* growth and development. In light of developing antifungal resistance, further study of *C. albicans* mt is important in that it may reveal unique therapeutic targets.
Chapter 3

CONCLUSIONS

1. The *C. albicans* *MspI* moderate molecular weight repeat units, ranging from 4.2 to 2.5kb, are mitochondrial in origin.

2. Mitochondrial clone lb13 (2.9kb) is specific to the mitochondrial compartment as determined by CHEF analysis. Mitochondrial clones lb10 (1.7kb), lb39 (1.4kb), and k1 (1.2kb) are likely specific to the mitochondrial compartment as evidenced by Southern analysis of restriction digests.

3. Dot blot hybridization analysis indicated that mitochondrial genome copy number increases over time during a yeast to hyphal morphogenesis. In addition, hyphal mutants showed greater increases in mitochondrial genome copy numbers than similarly grown wild-type strains.

4. Mitochondria undergo changes at the membrane level in addition to changes in number/volume during a yeast to hyphal morphogenesis as indicated by comparison studies of SDH and COX specific activity changes in cellular homogenates and crude mitochondrial fractions. My data suggest that mitochondria undergo changes in individual mitochondria prior to organelle replication/growth. Increases in mitochondrial numbers does not necessarily accompany increasing numbers of mitochondrial genomes early in the morphogenetic transition.
APPENDIX A

REPETITIVE DNA OF C. ALBICANS
Eukaryotic genomes are comprised of both unique and reiterated DNA sequences (42). The majority of unique sequences contain information coding for mRNAs while the reiterated component contains genes coding for rRNA, tRNA and mitochondrial DNA (346). Fungi are no exception and have been shown to contain both unique and repeated DNA sequences (35). In fungal systems, the majority of information regarding repetitive DNA was first described in research on *S. cerevisiae*, *N. crassa* (201), and *Aspergillus nidulans* (346). Recently, research has been extended to other fungal organisms (81), including *C. albicans* (see table 4). *C. albicans* has a genome with approximately 13% repetitive DNA (367). Although several repeat units have been defined, including mitochondrial DNA (309) and ribosomal DNA (219) repeat components, the biological function of dispersed, repetitive DNA families (301) and mitochondrial inverted repeats in *C. albicans* is not known.

Elucidation of the physical properties of DNA has facilitated genomic characterization, and the identification of repetitive DNA and unique DNA sequences. Separated complementary strands of purified DNA specifically reassociate under appropriate conditions such as cation concentration (i.e. Mg$^{2+}$, Ca$^{2+}$), temperature, incubation time, DNA size and concentration (42). The rate of reassociation can thus be determined by $C_0t$ analysis by altering DNA concentration and time of incubation (41). Eukaryotic DNAs contain different sequence classes as revealed by this kind of analysis, comprised of the following sequences: 1) unique (approximately one copy per haploid
genome), 2) repetitive (~10^3-10^5 copies per haploid genome), and 4) highly repetitive (~10^6 copies per haploid genome) (176).

Repetitive DNA has been well characterized in several different systems and includes Dictyostelium spp. (383), Xenopus spp. (74), and in more developed eukaryotes (239). Fungi have limited amounts of repetitive DNA (5 to 20 percent). Recently, determination of repeat units in C. albicans, and characterization of these repeats have been described (see table 4 for a summary of C. albicans repeats). In general, repetitive DNA consists of short sequences interspersed amongst single-copy DNA at short intervals (74), tandem arrangements (195), and inverted repeats (128,201,290,309). In addition, repeat units can be dispersed throughout the genome and can occur on several different chromosomes (50,81,294).

Table 4. Candida albicans repetitive sequences

<table>
<thead>
<tr>
<th>Repeat Unit</th>
<th>Length of Repeat</th>
<th>Possible function</th>
<th>Copy #/Genome</th>
<th>References</th>
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<td>1. rDNA</td>
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<td>Ribosomal</td>
<td>40-80</td>
<td>(219)</td>
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<td>2. mitDNA</td>
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<td>Mitochondrial</td>
<td>30</td>
<td>(367)</td>
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<tr>
<td>3. Ca-3</td>
<td>9.3 kb</td>
<td>N.D.</td>
<td>10</td>
<td>(294,328)</td>
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<tr>
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<td>9.5 kb</td>
<td>Telomeric</td>
<td>ND</td>
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<td>5. 27A</td>
<td>15 kb</td>
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<td>10</td>
<td>(303)</td>
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<td>6. pHpaCa7</td>
<td>0.33 kb</td>
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<td>&gt;6</td>
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<td>10. Alpha</td>
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Possible Functions and Applications of Repetitive DNA

Influence on Gene Control/Transposable Elements

In an early model of DNA repeat function, Britten and Davidson hypothesized that moderately repeated sequences of DNA may play a central role in gene regulation (40). Their model suggests that the mechanism by which unlinked structural genes are identified by the cell occurs via their linkage to repetitive sequence elements, as members of a coordinately acting gene set. The genome of Dictyostelium discoideum possesses a repetitive sequence that is flanked by different sequences depending on strain variation and is associated with a large set of developmentally regulated mRNAs. The position of this repeat supports the possibility for coordinated gene control and transposon-like function (63). Both control of mutation frequency by mating signals in yeasts (95) and gene control in Drosophila melanogaster (257) rely on transposable repetitive genetic elements.

Transposable elements are often characterized by the presence of direct or inverted terminal repeats and may be flanked by short repeats of DNA at the site of integration (288). A notable example of the transposable repeat elements is the Ty family (transposon terminal repeats) in S. cerevisiae. The Ty family is composed of three repetitive elements, delta and sigma, and tau (50). Ty1, a 5.9kb transposable element, affects the expression of certain genes located immediately downstream from the point of Ty1 insertion (95,114,287). In D. discoideum, a transposable repeat DIRS-1, is induced during differentiation and by heat shock. These inductions correlate with an increase in
transcript of approximately 20-fold during development compared with the amount of DIRS-1-specific RNA present in vegetative cells (383). Tdd-1, another repeat unit of *D. discoideum*, also is heat shock inducible and developmentally regulated (288). Transposons may, therefore, play a role in gene regulation in fungi.

Transposons in *Drosophila sp.* have been shown to be responsible for spontaneous point mutations (257). High copy numbers of P elements in transformation experiments suggests that this element encodes a transposase that mediates transposition of sequences lying between the terminal repeats. Ty elements have also been implicated in causing specific mutations and genetic instability (95,287,302). These findings indicate that transposons provide a mechanism for genetic variability in eukaryotic organisms.

**Transposable Elements and Genetic Instability in C. albicans.** Genetic plasticity in *C. albicans* is characterized by the presence of unpredictable chromosomal aberrations, including variations in chromosomal number and length (172,324). Transposable genetic elements can move from one site on a chromosome to another site on the same or different chromosome, thereby inducing DNA rearrangements (287). Transposons in *C. albicans* may explain the highly variable chromosomal structure of this yeast. UV-induced chromosome rearrangements and phenotypic switching in *C. albicans* often result in a high frequency of chromosomal alterations, which include additional chromosomes and changes in chromosomal lengths (23). A *C. albicans* transposon, Alpha, is a repetitive element that varies in both copy number and genomic location in several different strains (62). Alpha exhibits several features in common with
retotransposons, including conserved sequences within the long terminal repeats. Expression of alpha is strongly dependent on temperature. Other transposons in *C. albicans* may be linked to genomic instability and further investigation of repetitive DNA may provide information on the mechanisms responsible for genetic plasticity.

**Structural Repeat Units: Telomere and Centromere Association**

Centromeres and telomeres are chromosomal elements that are essential for stable inheritance of eukaryotic chromosomes (34). The centromere is a site of attachment to the spindle in mitosis and is required for the separation of homologous chromosomes in meiosis. A telomere is the region of DNA at the molecular end of a linear chromosome, and is required for accurate chromosomal replication and stability (34). In *C. albicans*, a dispersed repeat unit, Ca7, contains elements characteristic of telomeres, which includes tandem repeat elements and location at the most distal nucleotides of chromosome fragments (235).

Plasmids used in yeast transformation contain centromeric repeat sequences and autonomously replicating sequences (117,150,244,317). One critical feature of these plasmid systems is that these centromere-derived plasmids (CEN) are not completely stable. Plasmid loss and nondisjunction events are two commonly occurring events in yeast transformation (34). An effort to identify specific *C. albicans* centromeric and telomeric structural components yielded two repetitive sequences, Rel-1 and Rel-2 (345). The origin of these repeats has not been determined. The identification of centromeric and telomeric repeat sequences may be of use in creating stable plasmids in *C. albicans*. 
Taxonomical and Clinical Implications

The diagnosis of *C. albicans* infections is based on isolation and culture of organisms and the detection of circulating antigens and antibodies (28,48,109). While serological diagnosis of *C. albicans* is clinically significant, it poses several problems. To be of value, detection of serum antigens must be associated with disease rather than colonization, and detection sensitivity must be high enough early in the pathogenesis for antifungal therapy to be successful (258). Antigen must also be specific and not cross-react with antigens of other species. The detection of serum antibody may occur late in disease and may either be undetectable or absent, which would be of little diagnostic value (48). Patients may also demonstrate extensive invasive *C. albicans* infections of several deep organs, yet have negative blood cultures (90). These findings indicate that alternative methods of detection with increased sensitivity and a better correlation to disease should be investigated.

Recently, DNA typing schemes have offered an alternative solution to identify nosocomial outbreaks of *C. albicans* (228). In this study, restriction fragment length polymorphisms were used to type *C. albicans* strains. All isolates were typable within 48 hours. *C. albicans* strains can also be typed using electrophoretic karyotypes (356). The requirement of these methods involve the isolation of a significant amount of genomic DNA and the results can be difficult to interpret due to the large number of fragments. An alternative method of is to use species specific DNA probes. Several DNA probes have been identified that are *C. albicans* specific and are suitable for species identification (264) and strain identification (172). Although results of DNA probing are
easier to interpret than RFLP, the sensitivity of DNA probes to unique sequences is limited. DNA probes to repeated sequences may increase sensitivity levels.

Several repeated DNA sequences in *C. albicans* have been cloned and characterized in attempts to better understand the epidemiological and taxonomic relationships between species and among strains (209,303,304,327). There is currently no conventional genetic system for establishing genetic relationships in *Candida spp.* There is also no reliable system in which to differentiate between *C. albicans* strains (166). Repeat units demonstrating epidemiological and taxonomic potential rely on the presence of a stable and conserved repeat unit to observe minor changes over a period of time. The value of a repeat unit with these characteristics would allow the identification of evolutionary relationships between *Candida* species. In addition, a DNA probe to a conserved repeat unit would provide an epidemiological tool in which to determine strains specific to outbreaks such as nosocomially acquired *C. albicans* infections.
APPENDIX B

NORTHERN ANALYSIS OF pHpaCa7 S FRAGMENT
As discussed in Chapter 1, digestion of *C. albicans* total DNA with *Msp*I yields several putative high molecular weight repeats. Attempts to clone these *Msp*I repetitive elements into the *Cla*I site of pBR322 led to the report of a *C. albicans* specific DNA fragment (72). The recombinant plasmid, designated as pHpaCa7, was found to contain unexpected *Msp*I sites (72). *Msp*I digestion of pHpaCa7 yielded three fragments larger than similarly digested pBR322. These fragments were designated as L (large), M (medium), and S (small) and totaled 5.5 kb in length. The approximate amount of *C. albicans* DNA contained in the L, M, and S fragments are 3.8, 2.59, and .77 kb, respectively. Further characterization of these fragments by Southern analysis revealed that the L fragment is responsible for *C. albicans* specificity (122). In that study, the L fragment hybridized to *C. albicans* DNA, but not to DNA of other fungal or mammalian species. Conversely, both the M and S fragments hybridized to DNA of fungal and mammalian species. The S fragment proved to be of particular interest for the analysis of repetitive DNA sequences. Pulsed-field electrophoresis and Southern blot hybridizations showed that the S fragment probed to all of the CHEF-separated chromosomal bands. In addition, the S fragment hybridized to 14 different *Msp*I bands in an *Msp*I digest of *C. albicans* total DNA.

Northern analysis of the S fragment suggests that this DNA segment is transcribed (122). Blots of *C. albicans* total RNA were probed with 32P nick-translated S fragment, which hybridized to RNAs of 8.48 and 6.14 kb. In addition, dot blot analysis of total RNA was performed to determine expression levels in both yeast and pseudohyphae of *C. albicans*. Total RNA extracts from *C. albicans* strain 9938 pseudohyphae showed a five-
fold increase in mRNA levels over yeast cells. The expression levels of the S fragment were not determined for hyphal cells. As an extension of these studies, I chose to evaluate S fragment expression during a yeast to hyphal transition in *C. albicans* strain Cal. In this study, the production of hyphae was by growth in a serum-containing medium, and total RNA was extracted from cells at different time points during the morphogenetic transition. Hybridization analysis indicated that the S fragment was not differentially expressed during morphogenesis.

**RNA Extraction from *C. albicans***.

*C. albicans* yeast cells were grown in yeast nitrogen base (YNB) containing 2% glucose, and hyphae were induced in RPMI 1640 + 5% normal calf serum. RNA was extracted from *C. albicans* cells similar to methods described by Sundstrum et al. (341), at various time points in a yeast to hyphal transition. Time points are noted in the figure legend. Cell growth was stopped by the addition of cycloheximide at a final concentration of 150 µg/mL, and 5 additional minutes of incubation under the original culture conditions. Cells were rapidly cooled in an ice/water slurry and harvested by centrifugation. Cells were washed twice with 10mM Tris (pH8.0) containing 150 µg/mL cycloheximide and suspended in a solution of 50mM Tris (pH8.0), 1mM EDTA, and 200 µg/mL heparin. An equal volume of phenol was added to the cells, followed by the addition of glass beads at a weight twice the volume of the cell suspension. Cells were vortexed for 1 min and placed on ice. SDS was added to a final concentration of 0.5% prior to an additional 1min of vortexing. The supernatant was phenol/chloroform
extracted and the RNA was precipitated in 0.5M sodium acetate and ethanol. The final pellet was dissolved in certified pyrogen-free water and stored at -70°C.

**Northern Analysis of *C. albicans* RNA.**

*C. albicans* RNA samples were electrophoresed in 1.2% agarose gels containing 6.7% (w/v) formaldehyde and 1X running buffer (50X running buffer: 50mM EDTA, pH7.5, 0.5M NaH₂PO₄, 0.25M Na₂HPO₄, 0.4M NaOAc, pH7.0). Agarose was dissolved in 1X running buffer at 100°C and cooled to 50°C in a water bath prior to the addition of formaldehyde. Gels were poured and run in a fume hood. Gel buffer was 1X running buffer containing 6.3% formaldehyde (w/v). RNA samples for electrophoretic analysis were prepared by the addition of 8μl of RNA (1 μg/μl) to 20 μl sample mix (sample mix: 300μl deionized formamide, 90 μl formaldehyde, 12μl 50X running buffer). Samples were heated at 65°C for 15 min and cooled on ice prior to the addition of 1 μl ethidium bromide (1 mg/mL) and 1 μl bromophenol blue (10%). Gels were run for 2hrs at 80 volts and buffer was circulated with a peristaltic pump. Because ethidium bromide was added directly to the RNA samples, it was necessary to stop the gel at approximately 10min of run time and cut the unbound ethidium bromide from the top of the gel. Ethidium bromide has a net positive charge and will run opposite of the RNA. Failure to do this step will result in recirculation of the ethidium bromide to the opposite end of the gel and will mask the RNA bands when exposed to UV light. RNA agarose gels were blotted onto nitrocellulose membranes by capillary action (15) in 20X SSC, and RNA was affixed to membranes by baking at 80°C for 1hr in a vacuum oven. Probe purification,
radiolabeling, and hybridization methods and conditions were identical to those described in Chapter 2.

As shown in Fig. 7, the S fragment probe hybridized to a single RNA species of 4.8kb. Increased expression of the S fragment was not observed in C. albicans cells undergoing a yeast to hyphal transition. It is unclear why the hybridization was to a smaller sized RNA molecule than previously reported (122). One explanation is that the 4.8kb RNA was the result of degradation. This is unlikely because the 25S and 18s rRNA molecules appeared to be intact. These results confirm that the S fragment is expressed in C. albicans cells (122) and suggest that it is not differentially expressed during a yeast to hyphal transition. Further investigation is required to determine the origin of the S fragment.

Figure 7. Northern analysis of S fragment from C. albicans. (A) Total RNA was isolated from C. albicans cells in a yeast to hyphal transition and run on an agarose gel. RNA extracted from time 0 (Lane 1), 30 (lane 2), 60 (lane 3), 90 (lane 4), and 180min (lane 5) of germination. (B) Northern blot of gel from panel A probed with radiolabeled pHpaCa7 S fragment.
LITERATURE CITED


