Molecular genetics of a Candida albicans-specific DNA fragment
by Pati Maurene Glee

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

Abstract:
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Two other MspI/HpaII C. albicans DNA pieces were partially characterized by sequencing and both indicated positive hybridization to Northern blots of C. albicans RNA. One fragment showed potential for strain differentiation and multi-chromosomal hybridization.

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GLOSSARY OF TERMS AND ABBREVIATIONS

27A - *Candida albicans* mid-repetitive sequence used for RFLP

5AC - 5'-azacytidine

5AdC - 5'-aza-2'-deoxycytidine

5FC - 5'-fluorocytosine or 5'-flucytosine

5mC - 5'-methylcytosine

9938Y-Tx - 9938 yeast form RNA from CsCl method (DNAse treated)

ActS - spheroplast activation solution

APS - ammonium persulfate

ARS - autonomously replicating sequence

ATCC - American Type Culture Collection

B2 - RNA isolation buffer 2

B3 - RNA isolation buffer 3

B4 - RNA isolation buffer 4

Ca3 - *C. albicans* mid-repetitive sequence used for RFLP

Ca5 - *C. albicans* ribosomal DNA probe used for RFLP

Ca7 - *C. albicans* mid-repetitive sequence used for RFLP

cAMP - cyclic adenosine monophosphate

CAP - *C. albicans* secretory aspartyl proteinase

ccw - counterclockwise

cDNA - complementary DNA

CHEF - contour-clamped homogeneous electrophoretic fields

Comp-Tx - 9938 yeast form RNA from acidic phenol method (DNAse treated)

cw - clockwise

DEAE paper - diethylaminoethyl-modified cellulose paper for DNA isolation

DEPC-Tx - diethylpyrocarbonate treated

DNA - deoxyribonucleic acid

DigS - digestion solution

dITP - deoxyinosine-5'-triphosphate

dsDNA - double stranded DNA

DTT - dithiothreitol

EB1 - RNA isolation buffer 1

EF-1α - elongation factor

EPR - ethanolic perchlorate reagent

EtBr - ethidium bromide

EtOH - ethanol

FastA - a method of sequence similarity search for nucleic acids or proteins

FP - T7 forward primer

Geneclean - nucleic acid purification kit

GlcNAc - N-acetyl-D-glucosamine

GLS - genomic lysis solution

GYEP - 2% glucose, 0.3% yeast extract, and 1% peptone medium (complex medium)

HSB - high salt buffer

HSP - heat shock protein

IPTG - Isopropyl-1-thio-ß-D-galactoside

L - C. albicans DNA fragment which is the largest MspI piece in pHpaCa7

LINE - long interspersed repetitive sequence

LSB - low salt buffer

M - 2nd largest MspI piece from pHpaCa7 (part C. albicans and part pBR322)

MCID - microcomputer imaging device

MCS - multiple cloning site

mLee medium - modified Lee medium (defined medium). Lee medium contains various amino acids, salts, glucose, and biotin (1). Modified Lee medium contains sucrose instead of glucose, salts, and biotin, but no amino acids (2).

MOI - multiplicity of infection

mRNA - messenger ribonucleic acid

mtDB - mitochondrial DNAse buffer

mtDNA - mitochondrial DNA

mtGB - mitochondrial gradient buffer

mtWB - mitochondrial wash buffer

NTB - nick translation buffer

OD units - optical density units (MCID OD units were standardized)

OFAGE - orthogonal-field alternating gel electrophoresis

OLS - osmotic lysis solution

o/n - overnight

pBluescriptII SK (+ or -) - Stratagene phagemid cloning vectors code for ampicillin resistance, have a ColE1 origin of replication and the M13-related f1 intergenic region allowing ssDNA phage to be rescued by helper phage infection from F' bearing bacteria. pBluescript II vectors have a polylinker or MCS region flanked by T3 and T7 RNA polymerase promoters. The MCS region is present in the N-terminal portion of a *lacZ* gene fragment. Phagemids without inserts grow as blue colonies (eg., in XL1-Blue bacterial strain which has *lac*ΔM15 on an F' episome). pBluescript phagemids were constructed with both orientations of polylinker (SK and KS) and the f1 region (+ or -).

pBR322 - cloning vector carrying ampicillin and tetracyline resistance genes

pBSMsp-M22 - pBluescriptII SK(-) plus rearranged M fragment from pHpaCa7

pBSMsp-S9 - pBluescriptII SK(-) plus small MspI pBR322 piece

pBSPst+12 - pBluescriptII SK(+) with 1.2 kbp PstI piece from pHpaCa7

pBSPst+18 - pBluescriptII SK(+) with large PstI piece from pHpaCa7

PFGE - pulsed-field gel electrophoresis

pHpaCa7 - pBR322 vector with HpaII fragments from C. albicans 9938

pMCL-4 - pBluescriptII SK(-) plus largest ClaI x L subfragment

pMCS-1 - pBluescriptII SK(-) plus smallest ClaI x L subfragment

rDNA - ribosomal ribonucleic acid gene

RFLP - restriction fragment length polymorphism

RP - M13 reverse primer

RT - room temperature (21 - 24° C)

S - 3rd largest MspI piece from pHpaCa7 (part C. albicans, part pBR322)

SBB - slot blot buffer

SCE - sorbitol/sodium citrate/EDTA

SD - standard deviation

SEVAG - chloroform:iso-amyl alcohol (24:1)

SINE - short interspersed repetitive sequence

SSC - sodium chloride/sodium citrate

ssDNA - single-stranded DNA

SSPE - sodium chloride/sodium phosphate/EDTA

Sugelpot - DNA sample loading dye mix containing sucrose

TAE - Tris/acetate/EDTA electrophoresis buffer

TAFE - transverse alternating field electrophoresis

TBE - Tris/borate/EDTA electrophoresis buffer

VCS-M13 - Stratagene helper phage for pBluescript phage rescue

X-gal - 5-bromo-4-chloro-3-inolyl-ß-D-galactoside

XL1-Blue 2 - Stratagene Escherichia coli host for pBluescriptII SK(±)

YPD - 1% yeast extract, 2% peptone, 2% dextrose medium

◊I - doublet L fragment RFLP pattern (4.16 and 3.90 kbp)

◊II - singlet L fragment RFLP pattern (4.19 kbp)

◊III - singlet L fragment RFLP pattern (3.87 kbp)

◊IV - singlet L fragment RFLP pattern (3.58 kbp)

ABSTRACT

Candida albicans is an opportunistic fungal pathogen affecting increasing numbers of immunocompromised, at-risk patients. Efforts toward understanding *C. albicans* are focused on significant clinical questions concerning diagnosis, pathogenesis, and epidemiology of candidiasis, and also, developmental components and control of *C. albicans* morphogenesis. Molecular genetic studies hold promise for providing insight into this complex polymorphic organism.

The fungal cell wall acts as a barrier to extraction of high quality DNA for molecular analysis. In *C. albicans* studies reported here, Ribi Cell fractionation, autolysis, and spheroplast DNA extraction methods were equivalent at producing high molecular weight fragments that were susceptible to enzymatic manipulation. The distribution of MspI-digested (C↓CGG) *C. albicans* DNA contained unexpectedly large molecular weight fragments. Some MspI fragments larger than 4 kbp appeared repetitive by fluorescent staining intensity, but were not ribosomal or mitochondrial in origin.

Attempts to clone repetitive fragments led to the identification of a *C. albicans* and *C. stellatoidea* species-specific MspI/HpaII DNA fragment (Cutler, J.E., P.M. Glee, and H.L. Horn. 1988. J. Clin. Microbiol. 26:1720-1724). The 3.87 kbp *C. albicans* fragment did not hybridize to DNA from other *Candida* species including *C. krusei*, *C. tropicalis*, *C. kefyr*, *C. parapsilosis*, *C. guilliermondii*, or *C. lusitaniae*. Southern blot hybridization was extended to *S. cerevisiae*, *N. crassa*, *Torulopsis*, *Gaeumanomyces gramminis*, *Aspergillus*, *Mucor*, *Histoplasma*, *Sepedonium*, *Chrysosporium*, *Coccidioides*, *Cryptococcus*, *Blastomyces*, *Trichomonas vaginalis*, and to human and mouse DNA. The *C. albicans* specific fragment did not hybridize to any of these DNAs and may be useful as a DNA diagnostic probe. The *C. albicans* species-specific fragment indicated the following: 1 - 2 copies per haploid genome in DNA dot blots; 4 different MspI band patterns for 50 *C. albicans* isolates; no methylation changes at fragment-terminal CCGG sites during yeast to mycelial transition; partial sequence data revealed A-T rich regions; and positive hybridization to Northern blots of yeast and mycelial form RNA indicated transcribed sequences.

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INTRODUCTION

Clinical Significance of Candida albicans

Candida albicans usually lives as a commensal microorganism on mammalian mucosal surfaces, but can produce significant and life-threatening human disease. Among normal flora yeasts, C. albicans is regarded as having the greatest pathogenic potential (3). Alterations of immune function or changes in host physiology and resident flora can predispose individuals to opportunistic infections. Specifically, the use of immuno-suppressive therapeutic treatment of cancer and graft rejection, prolonged broad-spectrum antimicrobial administration, and inherited or acquired immune deficiencies, are contributing to increasing numbers of at-risk patients (3). Candidal infections, termed candidiasis or candidosis, can involve cutaneous and mucosal surfaces, subcutaneous tissue, or undergo hematogenous spread to develop lesions in multiple organs (4). Candidiasis advanced from an 8^{th} to a 4^{th} place ranking among nosocomial infections between 1984 and 1988, with occurrence of disseminated disease prolonging hospitalization by an average of 30 days (5).

Medically Important Candida Species

Demonstrated clinical significance exists for a small number of the nearly 200 Candida species which were taxonomically grouped as lacking known sexual states and growing primarily as unicellular

yeasts (6). Of the pathogenic species, C. albicans isolates are most frequently the cause of infection, followed by C. tropicalis, C. parapsilosis, C. krusei, C. stellatoidea, C. guilliermondii, C. lusitaniae, and C. kefyr (C. pseudotropicalis) (7,8). Some of these species now have a known sexual state (C. guilliermondii, C. kefyr, C. krusei, and C. lusitaniae) but name changes have been deferred because clinical isolates are usually the asexual stage (6).

In addition to traditional carbon source assimilation and fermentation profiles to distinguish between Candida species, C. albicans and C. stellatoidea exhibit dimorphic (technically polymorphic) growth as yeast forms, true hyphae, pseudohyphae, and chlamydospores (9). With the exception of very rare C. tropicalis isolates making germ tubes and chlamydospores, all non-C. albicans species make only pseudohyphae which display species-distinctive morphology also useful for differentiation (8). Because the carbon assimilation profiles and colony morphologies of C. albicans exhibit variation (described later), the rare occurrence of germinating C. tropicalis isolates may be due to inaccurate identification (8).

The specific status of the C. stellatoidea species has been debated because the isolates demonstrate few differences from C. albicans. One view incorporates C. stellatoidea isolates because they have 90% DNA homology to C. albicans, are able to produce hyphae and chlamydospores, and have just the classical distinction of negative sucrose assimilation (8). Two subtypes of C. stellatoidea isolates have been defined which differ in virulence and chromosomal karyotype (10,11) and are described in appropriate sections below. The

characteristics of Type I C. stellatoidea strains are viewed by some (11) as sufficient to keep separate species status. Use of the C. stellatoidea name in this work, reflects the fact that isolates had been characterized as Type I or Type II and was not intended to support separate speciation for C. albicans and C. stellatoidea.

Investigative efforts toward understanding the host-pathogen interactions, epidemiology, and basic biology of Candida species, primarily C. albicans, have increased in response to the current burden of candidiasis cases. Not only do significant clinical questions exist concerning colonization versus disease of mucosal surfaces, pathogenic virulence characteristics, diagnosis of deep-seated disease, and epidemiology of nosocomial infections, but also, developmental components and control of C. albicans morphogenesis remain undefined (8,12).

The principal focus for this dissertation dually includes assessment of DNA extraction procedures suitable for C. albicans genomic analysis and characterization of a C. albicans DNA fragment with indicated species-specific hybridization. Because additional topics are included in this dissertation (eg., methylation and repetitive DNA) the introduction will contain broad-based information concerning C. albicans. After descriptions of host-pathogen interaction, clinical considerations, and morphogenesis, the balance of the introduction will cover pertinent features of C. albicans molecular biology as background for experimental goals and design, and DNA analyses presented in the following sections.

Host-Pathogen Interactions

The interaction between C. albicans cells and host immunity has been reviewed from the perspective of host predisposing factors to candidiasis (8,13-19) and the virulence and variability characteristics of C. albicans (3,8,20-24). The following statements are not inclusive of all interaction phenomena, but provide a general description of primary features of the host-pathogen relationship.

Host Immunity

Under normal circumstances, host cell mediated immune responses and macrophages provide primary protection against opportunistic fungal pathogens. Defects or suppression of T-lymphocytes, phagocytic cells (25,26), or lymphokine production (27,28) have been identified as predisposing factors, allowing virulent invasion of host tissues by C. albicans [reviewed in (8,19)]. Although it has been shown that individuals colonized with Candida species produce antibodies recognizing Candida antigens, any protective effect remains uncertain because defects in antibody production are rarely associated with cases of candidiasis [reviewed in (15,16)].

Investigation of chronic mucocutaneous candidiasis has indicated various patterns of underlying T-cell defects, ranging from nearly complete T-cell unresponsiveness to different combinations of responses for mitogens, antigens, and Candida antigens [reviewed in (16)]. Such variation in host immune cell function has also been shown to result from immunomodulatory components of C. albicans cell wall mannan. Bulk preparations of Candida mannans contain a mixture

of components, some that incite normal antigenic responses and others that are non-antigenic and prompt suppression of responses to non-candidal antigens (20).

Physiological concentrations of ß-endorphin and prostaglandin E_2 (PGE_2) stimulate germ tube formation in C. albicans and may be involved in recurrent vaginitis (29,30). Women with recurrent infections have depressed cell-mediated responses to Candida antigens (31), have PGE_2 in vaginal fluid (32), and may have additional neuroendocrine signals which stimulate C. albicans morphogenesis (30).

Individuals with the highest risk of disseminated candidiasis have defective polymorphonuclear leukocyte (PMN) function such as neutropenia (12,19). Neutrophils offer the greatest candidacidal activity among phagocytic cells (28,33,34). In addition to iatrogenic factors such as surgery, immunosuppressive drugs, or antibacterial treatment mentioned before, other factors that can predispose the host to candidiasis are diabetes mellitus (35-37), adrenal or thyroid hormonal disorders (38), congenital diseases such as cystic fibrosis, or diet-associated factors such as high carbohydrate intake and vitamin deficiencies [reviewed in (8)].

C. albicans Virulence Factors

Among the cellular characteristics contributing to proposed pathogenic mechanisms of C. albicans are extracellular enzyme production, cell wall attributes such as adherence and antigenic variability, and possibly morphogenesis (21-23).

Enzymes. Extracellular proteinases and phospholipases made by C. albicans are expected to aid invasion of host tissue, but most have not been characterized in a definite virulence role [reviewed in (23)]. A secreted aspartyl proteinase has shown in vivo implications of conferring greater virulence for enzyme positive Candida isolates (39-47); but evidence eliminating either inter-isolate differences such as growth rate or possible multiple-hit mutagenesis lesions in proteinase-negative mutants has not been provided [discussed in (21)]. Recent cloning of the single Candida secretory aspartyl proteinase (CAP) gene by independent groups (48,49) will facilitate disruption mutagenesis of the CAP gene and virulence comparisons in mice of CAP-deficient mutants to parental wild type. Comparison of the first few N-terminal amino acids [full sequence only provided in one report (48)] indicates conservation at 11 of 15 residues for the CAP gene cloned from two C. albicans isolates. A third C. albicans aspartyl proteinase gene, *PRA1*, has been reported (50) which was 85% homologous to an internal proteinase of Saccharomyces cerevisiae, was significantly different from the other CAP gene reports (48,49), and probably doesn't code for a secreted enzyme from Candida.

Cell Wall Features. Physical interaction between host cells and C. albicans involves complexity not only of different host tissue surfaces (eg., epithelial mucosal surfaces, spleen, kidney, and lung), but also the variable cell wall surface of Candida. Recent reviews (8,22,51) cover in depth the various components of the Candida multi-regional wall ultrastructure: ß-glucans, mannans, mannoproteins, and small amounts of chitin. Factors including growth conditions, growth

stage, and morphogenic shape (yeast or hyphal form) are known to effect both the number of layers and relative composition of layer components (51,52).

Differences in the mannoprotein components, especially the mannosyl side chains that are the primary antigenic molecules of C. albicans, account for the two serotypes A and B (53). Numerous reports indicate that other surface antigens may vary in concentration or accessibility on yeast versus hyphal surfaces or between different isolates, growth stage, or nutrient sources (2,54-63). Such antigenic variability may contribute to virulence by evading specific immune recognition from the host; and as mentioned above, the mannans of Candida are capable of immunomodulatory effects (20).

The cell wall surface of C. albicans also contains specific receptor-ligand molecules that may contribute to virulence. Binding proteins for complement components and extracellular matrix proteins (laminin, fibronectin, and collagens) have been identified and may aid adherence of Candida cells to host tissue surfaces or target organs during hematogenous spread [reviewed in (21,22,64,65)]. C. albicans also exhibits general hydrophobic and hydrophilic cell surface differences which are influenced by growth temperature and growth stage (66,67). Cell surface hydrophobicity may promote attachment of Candida cells to human tissues and aid pathogenesis (66,68-71). The presence or accessibility of specific adhesins, such as one of the complement binding proteins (58), or general hydrophobic molecules (72) differ between yeast and hyphal form cell walls.

Morphogenesis. The ability of C. albicans to grow dimorphically has generally been accepted as a virulence trait because the presence of both yeast and hyphae (true hyphae and pseudohyphae) in infection lesions has been considered a hallmark disease indicator (3,8). Other than the cell wall associated antigenic, receptor, and hydrophobic differences between yeast and hyphal forms of C. albicans mentioned above, little experimental data supports the actual morphogenic shape change as a virulence characteristic.

Once engulfed by a phagocytic cell, yeast forms may be capable of germination and it has been suggested that they may escape phagocytosis by outgrowth of the hyphal filament (73-75). However, neutrophil competence in killing hyphal elements without engulfment has been shown (28,33,34). Any increased invasive potential of hyphal elements apart from the involvement of extracellular enzymes has not been defined, whereas yeast form invasiveness into keratinized skin and intestinal epithelium has been demonstrated and correlates with CAP enzyme production in C. albicans isolates (46,76-79). Demonstration that hyphal elements confer virulence advantages over yeast forms remains tentative.

Clinical Aspects of Candidiasis

Diagnosis

Traditional blood culture identification of C. albicans as the causative agent of disseminated disease remains problematic (8,80). Negative blood cultures for patients with extensive disseminated disease are common, as are positive blood cultures resulting from

transient candidemia (caused by infection of intravenous catheters) or just contamination from colonized body sites. Distinctions between colonization and disease by C. albicans are perplexing because surveillance culture recovery of yeast cells from peripheral body sites (urine, stool, or respiratory secretions) has very low predictive value for invasive infection (81). Current lysis-centrifugation blood samples, sometimes taken daily from at-risk patients, may require several days for Candida outgrowth to establish a positive culture (80,82).

Because of the insensitivity, non-specificity, and time - commitments of blood and surveillance culturing for the diagnosis of disseminated candidiasis, various alternatives are being tested for a faster, more accurate, and cost-effective specific test. Two main approaches designed to detect circulating Candida antigens or other fungal metabolic products have shown promise, but prospective clinical tests of detection assays indicate variable utility for diagnosing disseminated disease (81,83-85). A recent multi-center report (86) testing an immunoassay detection of circulating Candida enolase [a 48 kilodalton (kd) immunodominant antigen] (87-89) indicated an 85% sensitivity for deep tissue infection and 64% for fungemia when multiple sampling was done. These results appear encouraging that the enolase assay may aid detection when combined with blood cultures and/or other serologic tests (80).

Application of DNA probe technology to the generally difficult diagnosis of disseminated fungal infections has not been as productive when compared to other microbial infections or hereditary disorders

(90-93). The development of DNA probes for Histoplasma capsulatum (94), Blastomyces dermatitidis, and Coccidioides immitis have been suggested but details have not been published because of commercial pursuits (90). Some of the DNA fragments cloned from C. albicans have been used to distinguish between isolates of Candida species (49,95-98) and are described in the epidemiology section below. The species-specific DNA fragment reported in this investigation (99) exhibits hybridization only to C. albicans DNA which may indicate possible use as a diagnostic probe. Further comment regarding this point will be included in the discussion.

Antifungal Therapy

Currently, physicians will initiate antifungal treatment when high-risk patients develop fever of unknown origin and are unresponsive to antibacterial treatment (8,80). Disseminated candidiasis infrequently presents with fever, rash, or skin lesions, and usually doesn't demonstrate characteristic symptoms of infection. Therefore, empirical drug treatment reflects the need for aggressive therapy of suspected fungal infections when positive diagnosis may be too late: only 14-33% of patients have an antemortem diagnosis of disseminated Candida infections (81).

Amphotericin B has remained the most effective drug for disseminated candidiasis and other disseminated fungal infections since the 1950's (80,100,101). As a polyene compound binding to sterols, a narrow therapeutic index results from amphotericin B binding more tightly to yeast membrane ergosterol than to mammalian sterols (102). The binding event has been speculated to disrupt

plasma membrane potassium ion gradients, which are coupled to nutrient transport (eg., polyene treatment of Saccharomyces induces K^+ loss from the cells and inhibits maltose transport), and disrupt a proton-translocating adenosine triphosphatase found in the plasma membranes of Schizosaccharomyces, Saccharomyces, C. tropicalis and Neurospora (102). The hypothesized gradient disruptions suggest a mechanism for the broad range side-effects and toxicity seen in patients receiving amphotericin B (102). These side-effects include serious nephrotoxicity (experienced by over 80% of patients receiving the drug), anemia, anaphylaxis, generalized pain, chills, and fever (102). Nystatin, also a polyene, can be used for mucocutaneous infections, but is too toxic for parenternal administration (102,103).

A fluorinated pyrimidine, 5-fluorocytosine (flucytosine or 5FC), can be used alone or in combination with amphotericin B for disseminated Candida infections (100). Transported into cells, the compound becomes metabolized to 5-fluorodeoxyuridine monophosphate (dUMP), gets incorporated into RNA, and disrupts protein synthesis (102,104,105). Use of 5FC has been limited because C. albicans isolates frequently exhibit resistance (100,106,107). Substituted azole antifungals such as miconazole, itraconazole, and fluconazole are effective against mucocutaneous candidiasis (108). The azole compounds interact with cytochrome P-450 isozymes and likely inhibit ergosterol biosynthesis in Candida [reviewed in (102)].

Epidemiology

Investigating colonization versus disease, nosocomial outbreaks of candidiasis, isolate source for recurrent vaginitis, and identifying

different virulence characteristics among C. albicans isolates are significant epidemiologic tasks. Vigorous efforts toward providing greater isolate resolution than the two serotypes of C. albicans (53) have produced many typing methods including electrophoretic analysis of DNA for determining restriction length polymorphisms (RFLPs) among strains (95-97).

Biotyping Schemes. Described biotyping methods include resistogram typing (resistance to various chemicals) (109,110), morphotyping (variation in colony fringe and surface features) (111), and yeast killer typing (resistance to toxins made by S. cerevisiae, Ustilago, Kluyveromyces, and Pichia species) (112-114). Strain differentiation based on assimilation of carbon sources and production of extracellular enzymes has been used extensively in clinical and research laboratories. One biotyping scheme, developed by Odds and Abbott, uses a combination of resistogram and carbon assimilation tests (115,116) and has been used singly for epidemiology of Candida infections or in combination with DNA analysis (117,118). A recent review compared the reproducibility, typability, and discriminatory power (probability of a method to distinguish between two randomly chosen isolates from the population) of the various typing methods and indicated strongest support for DNA analysis over singular use of other biotyping methods (119).

DNA Typing Schemes. Strain differentiation by C. albicans DNA RFLP patterns has been done with electrophoresed EcoRI (G↓AATTC) or HinfI (G↓ANTC) digested DNA, that was stained with ethidium bromide

(EtBr) to show banding patterns (96,118,120,121). The brightest EcoRI and HinfI bands were identified as ribosomal DNA (rDNA) fragments that could be used to differentiate between medically important Candida species, S. cerevisiae, and Schizosaccharomyces pombe, either by EtBr-stained RFLPs or Southern blot hybridization to a C. albicans rDNA probe (Ca5 probe) (97). Two C. stellatoidea isolates included in the study were distinct from the C. albicans rDNA hybridization patterns (97). Mitochondrial DNA (mtDNA) probes from C. albicans showed HaeIII fragment polymorphisms, but these probes generate too few patterns for epidemiological use (119,122).

Many of the cloned fragments of (non-rDNA and non-mtDNA) repetitive DNA have been used as hybridization probes to distinguish Candida species and C. albicans strains. Middle-repetitive sequence Ca3 (approximately 10 copies per cell) provided stable patterns of hybridization for each strain of C. albicans, differentiated between strains, did not cross-hybridize with Saccharomyces DNA (123), and did cross-hybridize with C. tropicalis (98,124). Mid-repeat fragment Ca7 exhibited instability for individual strain typing (123) and has since shown hybridization patterns similar to S. cerevisiae telomeres; but Ca7 does not cross-hybridize to Saccharomyces, C. krusei, or C. glabrata DNA (98). Hybridization of Ca3 to strains isolated from cases of vaginitis indicated that multiple C. albicans isolates may be present during infection and that different isolates may be part of normal flora from various body sites (125). Another mid-repeat fragment 27A exhibited homology to Ca3 and distinguished between C. albicans strains, but produced a low rate of polymorphisms within the

tested strains (126,127). Fragment 27A was described as species-specific with no cross-hybridization to C. tropicalis or C. parapsilosis, but no other Candida species were screened (126).

Two gene sequences have been used for typing Candida species. Actin gene hybridization patterns differentiated between Candida species, except C. albicans and C. stellatoidea, and indicated conservation of EcoRI and SalI restriction sites flanking the actin gene for all C. albicans isolates tested (95). The secretory CAP gene cross-hybridized with C. tropicalis and C. parapsilosis genomic DNA under low-stringency Southern blots, but not under high-stringency wash conditions (49). Other Candida species, C. krusei, C. shehatae, C. guilliermondii, and C. lipolytica, did not show hybridization signals when probed with the C. albicans CAP gene DNA (49).

Caveats for C. albicans Epidemiology. The reproducibility of any typing method to identify an isolate depends upon stability of the tested traits, but some C. albicans isolates display high-frequency switching systems that affect colony morphology and phenotypic characteristics (123,125,128). Three colony morphology switching systems, described below, have been studied that display changes of features used in biotyping.

When C. albicans cells are plated onto amino-acid rich agar, smooth colony forms predominate, but wrinkled, star-shaped, ring, and other irregular profiles arise spontaneously at a frequency of 1.4×10^{-4} (128). Inter-morphology switches occurred in heritable and reversible fashion for some isolates, and the frequency of switching away from smooth form was elevated by ultraviolet (UV) exposure

(switch frequency = 2 x 10^{-2}, with UV survival at 90%) (128). Examining C. albicans colony morphology in plates of direct clinical samples confirmed different morphologies from the same isolate and confirmed that switching occurred at sites of active infection (123).

Another colony switching system indicated that 1.4% of C. albicans colonies, grown on Lee and Buckley medium which was designed to induce yeast to hyphal transition (1), spontaneously produced abnormal colony appearances (129). Two-thirds of the colony morphologies were stable but the rest switched rapidly into mixture colony phenotypes. Analysis of the mutant colony chromosomal karyotypes obtained by orthogonal-field-alternation gel electrophoresis (OFAGE) or transverse field (TAFE) gels and Southern blots of chromosomes probed with specific DNA markers, indicated that single and multiple chromosomal rearrangements had occurred (129).

White-opaque transitions of some C. albicans isolates occur at frequencies of 1 x 10^{-2} to 1 x 10^{-3} (119). Many cellular characteristics were found to switch heritably and reversibly along with the white-opaque colony switch. C. albicans in the white-form phenotype displayed normal ovoid cell shape, produced smooth colonies, and had other characteristics shown by the majority of clinical isolates (130,131). Opaque cells were elongate (twice the cell volume), produced flat opaque colonies (similar to some bacterial colonies), were more adherent to buccal epithelial cells (132), had pore-like openings through the cell wall (131), did not form hyphae in normal pH-regulated induction medium (described below) but did when attached to plastic or buccal epithelial cells (133), were more

resistant to polyenes and 5FC (134), and were unable to assimilate four carbohydrates when compared to the white-form phenotypic cells of the same isolate (125).

Low reproducibility of the morphotyping and carbon assimilation typing methods may be due to the high-frequency switching phenomenon (119). A relation between chromosomal karyotype rearrangements and DNA RFLP typing methods has not been determined. The use of more than one typing scheme (eg., DNA typing and biotyping) was recommended for strain differentiation and relating strains to phenotypic characteristics (118,119).

A tentative consensus view of C. albicans population structure (virulence differences, isolate distribution, and other physiologic parameters) indicates that most strains of C. albicans cluster within a range of physiological types which some reviewers regard as possibly preferred phenotypes (135-137).

Morphogenesis

Investigation of fungal morphogenesis is pursued for two major reasons: fungi provide simpler eukaryotic models for cell differentiation and gene regulation than mammalian systems; and, many fungal pathogens of man exhibit dimorphism that may relate to virulence [reviewed in (138)]. S. cerevisiae and Dictyostelium are two examples of fungi that been used to reveal information on eukaryotic gene promoter structure and transcriptional regulators (139-143), cellular cytoskeleton (144), stress (heat or nutrient) responses (145-148), transformation (149-152), cell cycle and

chromosomal replication (153-155), and cellular differentiation related cyclic nucleotide changes (156).

Fungal pathogens of man are divided into true pathogens, those that produce infections in normal hosts given a sufficient infective dose, and opportunistic pathogens that cause disease in immunocompromised individuals (3). Histoplasma, Blastomyces, Coccidioides, and Paracoccidioides are dimorphic true pathogens that undergo transition from filamentous forms at 25° C to grow almost exclusively as yeast (blastospores) at 37° C in human tissues. Common opportunistic pathogens include Aspergillus, Candida, Cryptococcus, Rhizopus, and Mucor. Of the opportunistic fungi, Candida albicans and Mucor are dimorphic, but only C. albicans regularly produces mixtures of blastospores, pseudohyphae, and true hyphae in vivo.

Much of the literature regarding fungal morphogenesis catalogues environmental factors which inhibit or stimulate morphologic changes and notes cellular changes of wall structure and components, cyclic nucleotides, pH, and enzyme activity. Techniques for DNA and RNA analysis are providing additional tools for monitoring changes of fungal gene expression which may be associated with dimorphism. Nevertheless, hypothetical models for morphogenic regulation which account for environmental stimuli, intracellular changes, and differential genetic expression, are generally lacking for fungal pathogens.

Morphogenesis in Other Fungal Pathogens

Morphogenesis in Histoplasma capsulatum, Blastomyces dermatitidis, and Paracoccidioides brasiliensis has been suggested to occur by analogous mechanisms because ultrastructural and biochemical changes in their temperature-regulated dimorphism are similar (138,157). Evaluation of the morphological changes has been done chiefly in H. capsulatum. Biotin and cysteine [supplying sulfhydryl (-SH) groups] are two nutritional factors required by H. capsulatum for growth and maintenance of yeast forms (157). Cyclic adenosine 3',5'-monophosphate (cAMP) levels increase five-fold when H. capsulatum yeast-forms convert to hyphae at 25° C; and in the presence of exogenous cAMP derivatives, yeast forms at 37° C will change to hyphae (158).

Analysis of differential gene expression possibly involved in H. capsulatum form transition has indicated some differences. Three α-tubulin messenger RNAs (mRNAs) are present in mycelia but only two are expressed in yeast (159). Also, a yeast-phase-specific gene, *yps-3*, was transcribed only in virulent H. capsulatum strains undergoing morphogenesis and not found in an avirulent Downs strain shifted to 37° C (94). Levels for heat shock protein (HSP) *hsp70* mRNA were lower for the Downs strain grown at 37° C than for virulent isolates, which may suggest a possible basis for lowered thermotolerance shown by the Downs isolate (157). The absence of a Downs' *yps-3* transcript was probably not due to *hsp70* regulation, because at 34° C *hsp70* mRNA levels in the Downs strain were similar to virulent strains while *yps-3* remained silent (160).

Mucor species produce filaments, sporangiospores, arthrospores, and zygospores like other zygomycetes, but can also make spherical, multipolar budding yeasts (161). Morphogenic transition in Mucor (hyphal to yeast) is regulated primarily by oxygen atmosphere and the concentration and type of hexose present: fermentable hexoses are required for growth as yeast forms; yeast forms predominate in anaerobic conditions; and, aerobic conditions induce hyphal phase growth [reviewed in (138,162)].

Analysis of mRNA from aerobic and anaerobic germination of Mucor sporangiospores indicates that stored pools of sporangiospore mRNA become rapidly translated to produce different protein patterns, and that no new RNA synthesis occurs during germination for at least 20 minutes in Mucor racemosus (161,163). Mucor has multiple actin, tubulin, ras, EF-1α (elongation factor), and MuR (possibly a transposon) genes that have each shown form-varying transcription levels [reviewed in (161)].

Morphogenesis in C. albicans

In vitro methods for promoting C. albicans yeast to mycelial transition involve changing nutrients, pH, or temperature (T). So far, no singular environmental condition has been identified that will promote germination for all C. albicans isolates (8).

True hyphae are distinguished from pseudohyphae by the absence of constrictions where the cylindrical hyphal element emerges from the mother yeast cell. Germ tube evagination sites can be any place on the surface whereas buds (blastoconidia) always form at polar regions, distal to the bud scar of the mother cell (8,51). Incorporation of

wall precursors occurs at the hyphal apical tip (164); and up until the first septal division, the one or two hyphal outgrowths per cell may be referred to as germ tubes. Hyphal length increases linearly with time and not exponentially as in other filamentous fungi (133,165-169). Hyphae can make lateral hyphal branches (37° C in serum) or lateral blastospores as hyphal cultures mature (40° C in peptone-containing medium) (8). Pseudohyphae are elongated blastspores, constricted at mother cell junctions and septal connections, and are also able to make lateral blastospores (24). Pseudohyphal length may appear like hyphae and may cause mistaken microscopic interpretation (except for the constrictions).

Induction Methods. Literature regarding C. albicans dimorphism contains many references of inter-isolate variation, contradictory effects of putative germination inducers, and growth phase or culture carbohydrate history differences that affect germination efficiency [reviewed in (6,8,24)]. Stating a few generalized parameters, though, will indicate common laboratory procedures for induction of C. albicans hyphal growth.

Yeast form cells grown in a defined medium (eg., amino acids/salts medium) remain as budding yeast cells at low T and high pH (T < 28° C, pH 6.5) or high T and low pH (T = 37° C, pH 4 - 4.5) (24). If blastoconidia grown in a defined or complex medium (eg., glucose/yeast extract/peptone medium) are placed in 37° C serum or buffer containing specific inducers [eg., N-acetyl-D-glucosamine (GlcNAc), L-proline, or glucose plus glutamine] most cells will germinate within 3 - 4 hours of incubation. Some isolates require a period of starvation (≥20

minutes) in order to germinate in the presence of specific inducers (170). C. albicans yeast cells grown at room temperature in defined medium (RT = 21 - 24° C, pH 6.5) will germinate if temperature-shifted into fresh medium at 37° C, or cells grown at pH 4.0 - 4.5 (37° C) germinate when pH-shifted to pH 6.5 (2.5 - 5 mM glucose required) (8,24). Of all the induction methods, hyphal formation in serum at 37° C provides the best consistency among isolates of C. albicans (8).

Non-germinating mutants of C. albicans have been described but generally have not proven stable (171-179). Some yeast-form mutants have low germination reversion frequency or will germinate in serum but not other induction methods; and hyphal-form mutants appear most like pseudohyphae which also make lateral blastospores [reviewed in (8)].

Factors Affecting Dimorphism. In addition to metabolic history (carbohydrate source) affecting germination, zinc concentrations from 1 μM to 3 μM promoted hyphal to yeast transition, but above 9μM Zn^{2+} the overall cell growth was inhibited (179-181). C. albicans also makes its own germination inhibitor, morphogenic autoregulatory substance (MARS), that suppresses dimorphism when cell concentrations exceed 1 x 10^6 cells/ml during induction (182-184). L-proline analogs (eg. sarcosine and L-axetidine-2-carboxylic acid) will suppress hyphal formation (185); but N-acetylmannosamine, a N-acetylhexosamine like GlcNAc, was able to induce the uptake system for GlcNAc and promote germination of C. albicans cells (170,186). Analyses of exogenous cAMP effects on germination and measures of intracellular cAMP levels have produced contradictory results (187,188). Growth of C. albicans

in elevated CO_2 promotes hyphal growth, similar to other dimorphic fungi [reviewed in (8)].

Associated Cellular Events. Induction by temperature, nutrients, or pH have caused the following intracellular biochemical changes: a) C. albicans internal pH rose from 6.8 to 8.0 during the first 30 minutes of induction and decreased to pH 7.0 prior to germ tube emergence (174,189); b) calcium ion channel blockers and calmodulin inhibitors blocked hyphal production (190-192); c) localized actin granules at the bud or hyphal cell wall evagination site remained confined to the apical tip of growing hyphae, but become dispersed along the growing bud cell wall (167,193); and d) chitin synthetase activity increased in germinating cells (164,194). The two modes of cell wall growth are referred to as general (bud growth) or apical (hyphal growth) cell wall synthesis (167).

Two dimensional electrophoretic analysis of yeast and hyphal form proteins of C. albicans have suggested more yeast-form specific proteins or amounts of proteins (ranging from 3 - 10 additional proteins depending on the report) and no hyphal specific proteins (195-197). Opposing reports indicated greater hyphal form amounts of 1 to 5 proteins but no yeast specific ones (198,199). Total numbers of resolved proteins varied between 200 to 400 in the various 2-dimensional patterns (8). Some of the cellular form-associated protein differences have been recognized and included with C. albicans stress responses (see below).

Eukaryotic and prokaryotic cells placed at elevated temperatures (5 - 15° C above culturing temperature) or in nutrient or chemical

stress respond by producing stress proteins which are either new or more abundant than unstressed cells. Because the germination induction methods for C. albicans also evoke stress responses, evaluation of the role of heat shock protein (HSP) responses to morphogenic processes has been considered [reviewed in (200)]. Comparison of temperature induced proteins produced by C. albicans germination-deficient mutants and normal hyphal cells indicated no major differences in nine HSPs produced at 37° C (18, 26, 34, 54, 72, 76, 85 and 98 Kda HSPs) (201). Nutritional stress proteins produced by germinating and germination- deficient cells were similar in the presence of specific inducers GlcNAc and L-proline, as were most other stress responses for conditions tested (200). No definite role for germination regulation has been observed for HSPs and related stress proteins.

DNA and RNA synthesis. DNA and RNA synthesis during yeast to mycelial transition has been studied, but the different culture conditions, isolates, and assay methods probably account for the diverse results. Two of the following investigations recorded increasing RNA and DNA during normal hyphal induction, whereas others reported hyphal growth without corresponding RNA increases or hyphal growth even in the presence of specific RNA and protein synthesis inhibitors.

Dabrowa, et al. (202), inoculated tissue culture medium (25° or 37° C) with stationary phase yeast and analyzed yeast and hyphal growth by hourly measuring dry weight, DNA content (by diphenylamine analysis (203)), RNA amounts (orcinol method), and protein content

(Lowry protein determination (204)). Yeast cell growth (25° C) exhibited no measurable changes at 1 and 2 hours; but at 3 hours the RNA had doubled (2x) and dry weight and protein increased (each 1.22x), but no increase in DNA content. At 4 hours, the yeast DNA, dry weight, and protein content was double (2x) the inoculum levels, and the RNA continued to increase (3.2x). C. albicans grown at 37° indicated change at 1 hour, but at the 2 hour sampling, 50% of the cells were germinating and displayed double RNA content (2x), increased protein (1.5x), increased dry weight (1.2x), but no increase in DNA amounts. At 3 hours with 95% germination, all measured values had increased compared to the initial inoculum levels: RNA (4x), DNA (1.5x), dry weight (2x), and protein (3x). No further increase in dry weight or RNA amounts were recorded at 4 hours, but DNA (2.2x) and proteins (3.1x) increased.

Wain, et al. (205) tracked DNA and RNA synthesis from singlet yeast cells (sucrose gradient prepared) inoculated into defined medium for yeast form growth (5% glucose plus yeast nitrogen base, 37° C) or into serum for hyphal form cells (37° C). Samples taken every 10 minutes for 6 hours indicated that DNA synthesis occurred in stepwise fashion (with lags of approximately 30 minutes) at the same rate and same time for yeast and hyphal growing cells. The RNA content increased immediately in both cell forms (no initial lag period like DNA), and the RNA in 2 hour-hyphal forms had increased (2.5 - 3x) when DNA had doubled (>95% germination). DNA amounts were assessed by the diphenylamine reaction and RNA amounts calculated from UV absorbance data.

In zinc-deficient medium, hyphae grew despite decreased RNA synthesis (RNA analysis by phosphorous colorimetric measurements) (181). 5FC treated C. albicans cells, which have inhibited RNA and protein synthesis, made hyphal filaments that were 80% of control length hyphae (105). Also, for cells germinated in buffered medium containing GlcNAc, the rate of protein synthesis increased within 30 - 60 minutes of induction and then rapidly decreased during 4 hours of normal hyphal growth (206). Decreased availability of external amino acids during the GlcNAc induction caused the rapid decrease in protein synthesis after 1 hour, but no changes in protein synthetic rates were observed when cells were temperature induced in Lee medium (206).

These various treatments to inhibit RNA synthesis during germination suggest that RNA synthesis is not required for hyphal production in C. albicans [reviewed in (8)]. Because the RNA quantifications, mentioned above, were done from bulk and not just mRNA levels, the measured decreases or inhibited RNA synthesis may reflect ribosomal RNA changes that could mask the small presence of other gene mRNAs. It would be informative to investigate RNA changes in normal germinating cells to identify biased production of rRNA or mRNA percentages.

Control of Morphogenesis. Substantial support for an environmentally regulated mechanism of C. albicans dimorphism has developed essentially because few proposed genetic regulators of cell wall morphogenesis have been observed. Because hyphal growth may be initiated in multi-path fashion, the synthesis and organization of cell wall components may be allosterically controlled and directed

without new gene expression. Such environmental modeling has been done extensively to explain biochemical differentiation of Dictyostelium, to reflect the complex interaction of structures and metabolic processes during dimorphism. Kinetic tracking of metabolites and altered enzyme activities have allowed investigators to identify 100 rate limiting substrates, inhibitors, effectors, and 40 interdependent enzyme reactions necessary for Dictyostelium aggregation [reviewed in (207-209)]. Dimorphism in C. albicans, at least for initial germination changes, may not depend on gene transcription but may occur through biochemical events controlling cell wall genesis (167;197,210).

Other data supporting environmental control has been discussed in above sections: paucity of phase specific proteins for normally germinating isolates or morphological mutants; hyphal extension (apical cell wall synthesis) despite RNA or protein synthesis inhibition; and, greater hyphal activity of chitin synthetase which is allosterically activated by GlcNAc [reviewed in (8,210)]. Various second-messenger pathways are likely involved because of Ca^{2+}-calmodulin activity or intracellular pH changes (mentioned above) and because of variable effects of germination inducers on different isolates. So far with DNA/RNA analysis just beginning, a six-fold increase in actin expression for germinating cells has been the only indication of possible genetic component to morphogenic regulation (211).

Cytoskeleton interaction with cell wall synthesis probably occurs through actin filaments and not microtubules in yeasts (122,144,210).

Cold-sensitive ß-tubulin mutants of S. cerevisiae carried out normal polarized growth and did not accumulate secretory vesicles when placed at restrictive temperatures; but actin mutants exhibited aberrant chitin deposition, accumulated vesicles, greater osmotic sensitivity, and eventual cell death during the budding portion of the cell cycle (210,212-214). Vesicle traffic along actin filaments occurred in a unidirectional fashion (interior to periphery), whereas microtubule-mediated vesicles may have bidirectional movement, depending on the associated transport proteins (212,215). Because bud and hyphal emergence sites are spatially different for C. albicans, the actin granule localization must be directed early in morphogenesis (210,216). Characterization of actin-binding proteins in S. cerevisiae have suggested differential association of the proteins to actin that may account for general or apical wall growth and some of the associations may be regulated by ATP (212).

Application of subtractive mRNA hybridization techniques to compare morphogenesis-associated changes in gene expression have been used for H. capsulatum (160), but tests with C. albicans have not been reported. Other possible regulatory contributors such as mRNA stability or translation rates have not been investigated, but may contribute to changes observed in the C. albicans 2-D protein analysis (as mentioned above).

Molecular Biology

Genome Characteristics

Because of the variability in morphology, cell surface, and other C. albicans physiologic traits, investigation of possible genomic

regulatory components has been searched. Over the last 10 years, application of genetic and molecular genetic techniques has furnished a limited foundation for understanding the C. albicans genome and provided specific tools to investigate possible genetic components of variability.

Diploid Genome. Establishing the diploid nature of C. albicans isolates was hampered by the asexual state and corresponding lack of normal sexual crosses used in classical genetics. Parasexual genetic analysis by mutant generation and selection, complementation by protoplast fusion (217), and observation of recombination events, provided early indications of diploidy [recently reviewed (218,219)]. Development of a transformation system for C. albicans supplied further evidence through disruption mutagenesis that some genes were present in two copies (see below) (220,221).

Mutagenesis of C. albicans by UV exposure, nitrous acid, or nitrosoguanidine resulted in high frequency isolation of auxotrophs from some strains. For example, when isolates were mutagenized, nearly 20% produced auxotrophs at a frequency of ≥0.01 per survivor, but other isolates yielded fewer auxotrophs (<0.001 per survivor) (222). Strains showing high frequency mutations also exhibited bias for particular lesions such as methionine, lysine, adenine, serine, leucine, histidine, arginine, or isoleucine - valine auxotrophy (223-227). The biased auxotrophic spectrum phenomenon has been referred to as "auxotypes" of C. albicans (218,222).

The occurrence of high auxotrophic frequencies from some C. albicans isolates suggested recessive alleles present in a naturally

heterozygous state (223). Polyethylene glycol fusion of auxotrophs was used extensively to interpret allele complementation groups and linkage relationships [(228,229), reviewed in (219)]. Seven linkage groups were determined, some containing recessive lethal and temperature sensitive loci (230,231). Resistance to 5FC was also indicated as naturally heterozygous, as 30% of clinical isolates showed slight resistance, 6% were highly resistant (probably homozygous), and the rest were susceptible to 5FC inhibition (106,222,232).

Transformation System. The first identification of a C. albicans gene sequence was the cloned orotidine-5'-phosphate decarboxylase gene (*URA3* gene) functioning to complement S. cerevisiae *ura3* and Escherichia coli *pyrF* mutants (233). An *ADE2* gene was next characterized by integrative transformation of a C. albicans adenine auxotroph (220).

Additional support for a diploid genome status came from disruption mutagenesis experiments that were combined with UV-induced recombination (221). The *ADE2* gene of C. albicans was inserted into the cloned *URA3* gene, and the plasmid construct used to transform a C. albicans *ade2* mutant. Integration of the plasmid at one resident *URA3* gene was confirmed by Southern blot. The adenine transformants were subsequently exposed to UV light and the resulting Ura- mutants had lost the second *URA3* resident allele through recombination events (221).

Various autonomously replicating sequences (ARS) from Candida have been functionally identified as improving transformation frequencies,

generated multiple plasmid copies per cell, and were unstable in the absence of marker selection (234,235). One characterized ARS sequence, CARS1, promoted formation of multimeric plasmids (234); whereas two other sequences, CaARS1 and CaARS2, have homologous structure and sequence to S. cerevisiae ARS regions near 5S rDNA (235).

C. albicans isolates exhibit high natural resistance to benomyl, methotrexate, G418, and hygromycin B, so other markers are being sought for dominant selection of transformants (127,152,236,237).

Nuclear and Mitochondrial DNA. The relative percent G + C nucleotide content in C. albicans DNA has been reported as 32.8% for nuclear and 38.2% for mitochondrial DNA (mtDNA) as determined by analytical cesium chloride (CsCl) gradient analysis (238). Eight reported estimates of C. albicans DNA content cluster between 37 - 40 femptograms (fg) per cell, indicating approximately 36 to 39 megabase pairs (mbp) of DNA in the genome [reviewed in (127,239)].

Ribosomal DNA (rDNA) repeats of C. albicans have an approximate length of 12 kbp (97) and are present at 40 - 80 copies per cell (0.48 to 0.96 mbp rDNA), estimated roughly from tandem length intact rDNA, electrophoresed on pulsed field gels (127). Subcloned fragments from the C. albicans 18S and 25S rDNA gene regions have been used to draft restriction maps of C. glabrata, C. tropicalis, and C. guilliermondii rDNA units and for distinguishing between Candida species (discussed above) (97). The rDNA repeat number for the 9.1 kbp unit of N. crassa varies in wild-type strains from 185 (74-OR23-1A, Oak Ridge-derived)

to 225 (AR33) (240,241). Saccharomyces has approximately 100 copies of 9 kbp rDNA repeat per haploid genome (242).

Using purified mitochondria, Wills, et al., have estimated mtDNA size at 40 kilobase pair (kbp) by EcoRI and PvuI (CGAT↓CG) restriction digests length and from electron micrographs of platinum-palladium shadowed nucleic acids (including ϕX174 phage DNA as internal standard circles) which also indicated circular molecules of C. albicans mtDNA (238,243). The six EcoRI mtDNA fragments have been subcloned for mitochondrial gene mapping experiments, for RFLP differentiation of C. albicans isolates (mentioned above in the epidemiology section), and for estimating approximately 30 mtDNA copies present per cell (30 x 41 kbp = 1,230 kbp mtDNA) (127,238,244,245).

There are three general classes of fungal mitochondrial genes: components of mitochondrial protein synthesis (rRNAs, tRNAs, and usually one fungal ribosomal subunit protein such as *var1* in S. cerevisiae); components of ATP synthetase complexes like cytochrome oxidase and cytochrome *b*; and, sequences containing unassigned reading frames that constitute most of the species-associated mitochondrial genome differences [reviewed in (246,247)]. In S. cerevisiae two unassigned reading frames occur in cytochrome *b* introns and code for RNA splicing maturases (246). Incidences of genes (ATPase subunits, cytochrome oxidase, and rDNA genes) or A-T rich DNA sequences shared by nuclear and mitochondrial genomes have been identified for Saccharomyces, Neurospora, Podospora, Locusta, and Strongylocentrotus (sea urchin) (246,248-251).