MONTANA STATE UNIVERSITY

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
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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 (ClCGG) 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. Gaeumanomvces gramminis. Aspergillus, Mucor, Histoplasma. Sepedoniurn, Chrysosporium. Coccidioides, Cryptococcus, Blastomyces. T richomonas 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.

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.

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.

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).

DNA Complexity. Riggsby et al., analyzed C. albicans DNA kinetic complexity by calculating reassociation rates in the C_0t method of Britten and Kohne (252,253). Whole-cell DNA samples were sheared (average size 300 bp), heat dissociated, allowed to reassociate for varying lengths of time, and double-stranded DNA (dsDNA) concentrations were analyzed by hydroxylapatite chromatography, S1 nuclease digestion, and optical hypochromicity (decrease in UV absorption occuring as dsDNA forms from annealing single strands).

C. albicans DNA expressed two component reassociation kinetics with 13 - 15% repetitive and 85% single copy sequences (252). Further analysis of just the repetitive DNA fraction indicated approximately 13% was a fast reassociation component and 7.1% was a slow component by comparison of observed reassociation constants (238). Riggsby, et al., calculated the reiteration frequency per haploid genome to be 17 copies of 74.8 kbp slow components and 280 copies of 1.3 kbp fast components (238).

These C. albicans values are similar to S. cerevisiae (3.2×10^7 bp genome, 13% repetitive, and 87% single copy), Neurospora (2.7×10^7 bp genome, 10% repetitive, 90% single-copy), and other lower eukaryotes except Aspergillus nidulans (2.6×10^7 bp genome, 2 - 3% repetitive, and 97 - 98% unique sequences) for DNA complexity and the amount of repetitive sequences per genome (127,252,254-256). The slime mold Dictyostelium discoideum has approximately 30% repetitive DNA similar to the level of repetitive sequences in mammalian genomes (253,257,258). Plant genomes are considerably more repetitive, with

values of 50% common; a notable exception being Arabidopsis which has only a 7 x 10^7 bp genome and little repetitive DNA (259).

Riggsby, et al., did not determine the organization pattern of C. albicans repetitive fragments which has been done for other organisms to indicate associations to single-copy sequences and speculate function for repetitive DNA (260,261). For example, tandem arrangement of rDNA genes or satellite DNA sequences, which are multiple copies of simple divergent repeats, would suggest limited interaction with single-copy sequences (258,261,262). Dispersed repetitive sequences such as mammalian short interspersed (SINEs are <500 bp, 10^5 repeats) or long interspersed (LINEs are >5 kbp, 10^4 repeats) elements are transcribed in some cases (263-265) and a rat LINE element functioned to promote chloramphenicol acetyltransferase gene expression in a rat cell line (266). The rat LINE element promoter region contained 22 CpG dinucleotides and lost promoter function when only 5 sites were modified by methylation of the cytosine residues (266). In general, though, specific functions for the dispersed repetitive DNA sequences remain speculative and may not exist (267). (More on CpG methylation included in Appendix A.)

Karyotype of C. albicans. Soon after separation of S. cerevisiae chromosomal DNA by pulsed field gel electrophoresis (PFGE) and OFAGE (268,269), C. albicans became a "next-step" organism to test electrophoretic parameters for separating larger DNA molecules. PFGE indicated 5 - 6 poorly resolved C. albicans chromosomes (270). Vollrath and Davis (271) introduced contour-clamped homogeneous electric field (CHEF) gels capable of 6 - 7 band resolution, showing

that the smallest Candida chromosomes (about 1.2 mbp) are the size of the largest S. cerevisiae ones, and that the largest Candida chromosomes (approximately 5 mbp) approach S. pombe chromosomal sizes.

Different isolates of C. albicans exhibited variation in separated chromosomal band sizes (EtBr staining and chromosomal specific probing) and the patterns appeared different from other medically important Candida species (272-274). Two additional levels of variability for C. albicans chromosomal configuration have been noted. Southern blots of C. albicans chromosomes probed with *ADE2*, *HIS3*, or *URA3* genes indicated signals from resolved bands that suggests size differences in homologous chromosomes (272,273). And, as was described above, the karyotype for some C. albicans isolates undergoing switching colony morphology can exhibit differing chromosomal migration (129). Clinical isolates displaying normal colony morphology also showed similar migration differences; for example, gene probes for the largest chromosomes showed differing size and relative position for various C. albicans isolates (127,273). In spite of variations, karyotype investigators have agreed that most C. albicans isolates have 8 chromosomes with the best resolution occurring on TAFE gels (98,127,236,275) or OFAGE and FIGE (276).

C. stellatoidea isolates displayed the same range of DNA content per cell as C. albicans (277) and those examined for chromosomal karyotype distributed into two groups: Type I - exhibited chromosomal polymorphisms with 9 - 11 separated bands which included some smaller chromosomes than seen among C. albicans isolates; and, Type II - displayed 8 bands like the majority of C. albicans (10,11). Type I

isolates had lower virulence, produced proteinase at low pH, and had slow growth rates on cycloheximide-bromcresol green agar; whereas, type II isolates exhibited normal virulence (similar to low virulence C. albicans isolates), no proteinase at low pH, greater resistance to low UV irradiation, and normal growth rates on the indicator medium (10).

Cloned Genes. Most of the genes cloned from C. albicans have been identified either by complementation of S. cerevisiae mutants or by S. cerevisiae gene probes of C. albicans genomic or complementary DNA (cDNA) libraries. Screening with a heterologous system, though, has been unsuccessful for selecting some C. albicans genes, indicating obstacles similar to ones encountered by using S. cerevisiae for Aspergillus and Neurospora gene identification (278). Recent reviews have listed the 35 - 40 C. albicans genes for biosynthetic enzymes, cell membrane or cell wall synthesis enzymes, structural or cell cycle genes, carbohydrate utilization and protease genes (122,127,236).

Limited information regarding C. albicans gene structure comes from only a few sequenced genes. Preliminary evaluation indicates similarity to RNA polymerase II type regulatory elements of Saccharomyces promoter regions (236). Eight of 11 Candida genes have conserved TATA boxes (Hogness box = TATAAA consensus sequence) upstream of the ATG transcription start codon (48,236). TATA sites are recognized by TFIID, a eukaryotic protein similar in function to prokaryotic sigma transcription factors which interact with RNA polymerase II (279). The genes without consensus TATA boxes may have diverse sequences functioning as translational activators such as

those shown in S. cerevisiae and may occur over a greater sequence range (40 - 120 bp upstream) (139,280). Unlike mammalian promoters usually displaying a single site for transcription initiation that is 25 - 30 bp from a TATA box, some S. cerevisiae promoters show multiple initiation sites for a single gene when multiple TATA elements are present (eg., cytochrome c gene transcription occurs at +1, +10, +16, +25, +34, and +43 with 4 upstream TATA sequences) (139,281).

S. cerevisiae RNA polymerase II promoters have two other main functional elements besides TATA sequences. Upstream activating sequences (UASs) are usually 10 - 30 bp long, may be at variable distances from the mRNA initiation site, and consist of different DNA sequences depending on the gene (139). UAS elements are analogous to the enhancer elements of mammalian promoters such as the CCAAT box and GC box (the latter GGGCGG sequences are bound by the sequence-specific transcription factor, Sp1) (139,282). Initiator elements, the third type of yeast promoter elements, are found near the actual mRNA start site and they are a primary determinant of where transcription begins (139). The spacing between initiator elements and TATA boxes in yeast promoters exhibits greater flexibility than mammalian promoters (139).

No analyses of C. albicans 5' promoter regions have included identification of UAS or initiator elements [reviewed in (236)]. The C. albicans actin and ß-tubulin genes have introns with conserved branch and 3' splicing signals for mRNA processing analogous to S. cerevisiae (95,283). Some C. albicans genes express codon bias (42 to 49 codons of possible 61) like constitutively expressed genes of

S. cerevisiae (*PRA1, TEF1, TEF2, TUB2*), whereas other genes do not (*ERG16, CDC10*) (236).

mRNA Translation. Recently, an mRNA-dependent cell free translation system was reported by Colthurst, et al., which provides another molecular tool for analysis of Candida transcripts (284). Their results indicated that C. albicans lysates, prepared from glass bead disrupted cells, exhibited translational characteristics similar to Saccharomyces lysate systems. Translation of poly(U) templates by Candida lysates was efficiently done in a template- and Mg^{2+}-dependent manner, and translation events exhibited strict requirement for ATP. Micrococcal nuclease treated lysates (free of endogenous mRNA templates) were capable of translating exogenously supplied homologous and heterologous (S. cerevisiae) templates. Electrophoresed proteins, produced by the C. albicans cell free lysates, indicated that large proteins (above 100 kDa) were synthesized.

Antibiotics known to inhibit specific steps of translation elongation in Saccharomyces lysates also inhibited the Candida systems: tetracycline and fusidic acid that target aminoacyl-tRNA binding to ribosome subunits; puromycin targets peptide bond formation; and emetine and diphtheria toxin (but not cycloheximide) target ribosomal translocation. Candida lysate resistance to cycloheximide inhibition correlates with the observed cycloheximide tolerance of Candida isolates (8).

The C. albicans cell free translation system also exhibited the same temperature sensitivity as Saccharomyces lysates and would not translate exogenous natural mRNAs above 20 - 23°C, though poly(U)

templates were translated efficiently at 30° C or 37° C in both yeast systems (284,285). The temperature inhibition of S. cerevisiae lysates has been shown to be reversible and probably not due to a temperature-activated nuclease because 5 - 10 minute incubation of extracts at 37° C, followed by transfer back to 23° C, allows 75% recovery of protein synthesis rate (285). The temperature sensitivity and above mentioned strict requirement for ATP are two characteristics of the yeast systems which differ from other eukaryotic cell free translational system (120,284,286).

Investigation Background and Goals

At the onset of this project, very little information was available on the molecular biology of C. albicans. In addition to some of the parasexual fusion and auxotrophic linkage information, only the DNA kinetic complexity study (252) and purification of mitochondrial DNA had been done (238). As was mentioned earlier, one goal for the following experiments included evaluation of DNA extraction procedures for providing high quality DNA from C. albicans cells for molecular genetic or RFLP analysis with enzymes for RFLP analysis and other DNA manipulations such as construction of genomic libraries.

DNA Extraction

Methods for extracting high quality fungal DNA involve enzymatic or mechanical breakage of the fungal cell wall. For S. cerevisiae, cell wall digestion has been done with various enzymes: lyticase, glusulases (which may contain DNAse activity), helicase from Helix

pomatia, chitinase, and Zymolyase from Arthrobacter [reviewed in (287,288)]. DNA isolation usually involved osmotic lysis of the spheroplasts, proteinase treatment, and organic extraction of the preparation; and estimates of Saccharomyces DNA fragment sizes after spheroplast extraction were 10 - 20 kbp (28,289).

Observation of the differential susceptibility of various yeast genera to different enzymes and treatment conditions has been ample (28,290- 292). C. albicans was similar in that pretreatment of cells with dithiothreitol and proteinase was necessary before helicase (glucuronidase) and chitinase digestion efficiently produced spheroplasts from all phases of growth and yeast and mycelial forms (290,293). Spheroplasts prepared with the pretreatment were used for mitochondrial purification and whole cell DNA extraction, but no estimates of extracted fragment sizes were given (238). Zymolyase, which contains glucanases, mannanases, some proteinase activity, but no DNAse or RNAse activity, had been used for removal of C. albicans cell walls in protoplast fusion experiments, and also to selectively remove cell wall components for structural analysis (2,294).

Mechanical release of fungal nucleic acids has been done by various techniques. Vortexing with glass beads of small diameter (eg., 0.45 mm) has produced sheared, low molecular weight DNA fragments which may be used for kinetic studies or RNA samples (260,261,295,296). Passage of cells through high-pressure (French Pressure Cell) apparatus provided high molecular weight DNA from Neurospora (240). An autolysis method, that involves vortexing a lyophilized fungal biomass with 4 mm glass beads and subsequent

incubation in lysis buffer (containing Triton X-100 and proteinase) has also provided high molecular weight DNA from Neurospora (241).

Comparison of DNA Extraction Methods. Comparison of various DNA extraction methods for C. albicans was initiated as a result of two initial observations. Electrophoresed restriction digests of C. albicans DNA, whether from a six-base (238) or four-base recognition enzyme, produced brightly staining EtBr bands suggesting repetitive DNA sequences ((297), and unpublished observations of J. Cutler and P. Russell). Cutler and Russell's work with rDNA hybridization to Candida DNA indicated that only some of the putative repetitive sequences could be attributed to rDNA repeats and that comparison of different isolates by the digest band patterns may be possible.

In addition, the MspI (C↓CGG) digested C. albicans DNA exhibited high molecular weight fragments which were not seen in similarly digested Saccharomyces or Neurospora DNA samples. C. albicans and Saccharomyces have roughly the same amount of DNA per haploid genome and similar kinetic complexity (256), but the distribution of MspI fragments differed in a way suggesting longer A-T rich segments or methylation protection of possible CCGG sites in C. albicans.

Further RFLP analysis of such intra-strain banding patterns would require quality DNA preparations (high molecular weight fragments and susceptibility to restriction enzyme digestion) from multiple isolates of C. albicans. In their initial studies, Cutler and Russell applied the French Pressure Cell and autolysis extraction methods from Neurospora to prepare the Candida DNA samples. A comparison of DNA extraction methods was initiated and included analysis of C. albicans

cell susceptibility to Zymolyase cell wall digestion and the quality of DNA extracted from spheroplasted cells. Zymolyase was chosen because of the absence of DNAse and RNAse activity in commercial preparations. Results of comparing DNA quality from the various extraction methods has been previously published (298) and are included in the following sections with exception of the autolysis method. Post-publication, the suitablility of Zymolyase digestion upon hyphal form C. albicans for subsequent DNA extraction was tested and the results are included here.

Characterization of a C. albicans Species-Specific DNA Fragment

The second goal of this investigation was to further characterize a cloned HpaII-digest C. albicans fragment which had been provided by J. Cutler and H. Horn. The fragment appeared to be of similar size to bright MspI or HpaII bands suggesting that the fragment may be repetitive. When the fragment was radiolabeled and used to probe digests of various C. albicans isolates different sized signals were observed. Also, preliminary screening of DNA from some other Candida species, other fungi, and mammalian DNA indicated that the C. albicans fragment did not hybridize with other species and may be a candidate for a diagnostic probe. The above observations of fragment characteristics were previously published and included a tentative restriction map (99).

The specific goals for examining the putative species-specific fragment included the following: extended survey of DNA hybridization to include other medically important fungi; develop a restriction map

of the fragment and determine the copy number per cell; survey various C. albicans isolates for intra-strain RFLP patterns; investigate possible methylation changes at the terminal CCGG sites of the fragment; determine nuclear or mitochondrial origin of the fragment; examine RNA extracts to indicate if the fragment or portions of the fragment were transcribed in yeast form or hyphal form cells; and, to obtain sequence information for the putative species-specific fragment.

MATERIALS AND METHODS

Materials

Organisms and Culture Conditions

Candida albicans strains maintained in our laboratory stock collection are clinical isolates. Strains B23578, Sh27, and H12 were obtained from D. Stevens (Santa Clara Valley Medical Center, Palo Alto, CA). *C. parapsilosis* isolates were gifts from M.G. Rinaldi (Audie Murphy Memorial Veterans Hospital, San Antonio, TX). *C. albicans* ATCC 32354 and *C. stellatoidea* ATCC 36232 were from the American Type Culture Collection (Rockville, MD); while *C. stellatoidea* ATCC 4252 and ATCC 4365 were gifts from K.J. Kwon-Chung (NIH, Bethesda, MD). Other *Candida* species and fungal isolates were from the Montana State University stock collection. Species identification was reconfirmed with yeast identification strips (API 20C, Analytab Products, Plainview, NY). Germ tubes were produced by all *C. albicans* isolates tested. Stock cultures were maintained on refrigerated slants of Sabouraud-dextrose agar or potato flakes agar (299), and -20° sterile distilled H_2O (d-H_2O) stocks or -70° C glycerol stocks.

Yeast-form *C. albicans* were grown in GYEP broth (2% glucose, 0.3% yeast extract, 1% peptone) at 37° C or modified Lee Medium (mLee: 0.5% NaCl, 0.5% $(NH_4)_2SO_4$, 0.02% $MgSO_4$-$7H_2O$, 0.25% K_2HPO_4), and 1.0% sucrose with filter-sterilized biotin added to 0.04 mg/l at room temperature (RT

= 22 - 25^0 C) with shaking (160 rpm) for 24-48 hours (h). Cultures for temperature shift induction of mycelial cells were grown in mLee for 24 - 48 h (2), centrifuged and washed in sterile dH_2O before re-inoculated at a cell density of 5 x 10^5 - 5 x 10^6 cells/ml into fresh mLee containing 1% bovine serum albumin (BSA). Germination was monitored microscopically up to 4 h after induction. Saccharomyces cerevisiae cells were grown in YPD broth (1% yeast extract, 2% peptone, 2% glucose) at RT to log-phase or stationary phase for DNA extractions.

Escherichia coli strains used for transformation were DH5α (*F-, endA1, hsdR17, supE44, thi-1, recA1, gyrA86, relA1, 0801acZΔM15*), provided by Joan Henson (Montana State University) and XL1-Blue 2 (*recA1, lac-, endA1, gyrA96, thi, hsdR17, supE44, relA1, [F', proAB, lacI^q, lacZΔM15, Tn10]*) from Stratagene (300). The XL1-Blue 2 cells were routinely grown in the presence of tetracycline (12.5 µg/ml) to retain the F' plasmid. Bacterial cells were prepared by the $CaCl_2$ method for transformation (296) [p. 1.82-1.84]. Log phase cells [OD_{540nm}= 0.2-0.4, 50 ml Luria broth (LB) culture] were incubated in 25 ml 0.1 M $CaCl_2$ for 25 min on ice, pelleted (3000xg, 15 min), and suspended in 5 x 100ul 0.1 M $CaCl_2$ aliquots. Competent cell aliquots were incubated with the appropriate DNA 1 h on ice, heat shocked 1 min at 42^0 C, diluted into pre-warmed LB and grown for 2 h at 37^0 before plating on appropriate solid media.

The C. albicans actin gene plasmid (95) was a gift from W. Stuart Riggsby (University of Tennessee, Knoxville, TN), a plasmid carrying the C. albicans ura3 gene was a gift from Donald R. Kirsch (Squibb Institute for Medical Research, Princeton, NJ), and a N. crassa rDNA containing

plasmid was a gift from Peter J. Russell (Reed College, OR)(221,233). Various fungal and protozoan DNA samples that were graciously donated for hybridization surveys are indicated in Table 1.

Table 1. Donated fungal and protozoan DNA.

Organism	Source
Gaeumanomyces graminis vars. graminis, tritici, and avenae	Joan Henson, Montana State University
Neurospora crassa 74-OR-23-1A	Peter J. Russell, Reed College
Cryptococcus neoformans H99	John R. Perfect, Duke University
Coccidioides immitis	Gary T. Cole, University of Texas
Mucor racemosus 1216B	John E. Linz, Michigan State University
Aspergillus flavus H125	David M. Geiser, University of Georgia
Trichomonas vaginalis S-981	Don Riley, University of Washington
Histoplasma capsulatum G222B ATCC26034, Blastomyces dermatitidis CR, Sepedonium chrysospermum ATCC16084, Chrysosporium keratinophilum ATCC 14803	Elizabeth J. Keath, Washington University (94)

Reagents, Enzymes, and Cloning Vectors

Reagent grade liquid organic chemicals and other chemical reagents were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ) and Sigma Chemical Co. (St. Louis, MO), unless specifically noted. Zymolyase 20T was purchased from Miles Scientific (Naperville, IL) or ICN Biomedicals, Inc. (Irvine, CA). Radioisotopes, scintillation fluid, and GeneScreen Plus hybridization transfer membrane were purchased from NEN Research Products (Boston, MA). Transblot Transfer Membrane (DEAE Ion Exchange Paper) was purchased from Bio-Rad (Richmond, CA), the Geneclean Kit (silica gel DNA-capture matrix) was from Bio 101, Inc. (LaJolla, CA), and the DNA Dipstick quantification kit was from Invitrogen (San Diego, CA). Genetic technology grade NuSieve GTG, SeaKem GTG and SeaPlaque agaroses were obtained from FMC Bioproducts (Rockland, ME).

DNA restriction endonucleases and modifying enzymes (polymerases, phosphatase, and ligases) were obtained from Stratagene (LaJolla, CA), United States Biochemical Corp. (Cleveland, OH), BRL Life Technologies (Gaithersburg, MD), Promega Biotec (Madison, WI), New England Biolabs (Beverly, MA), and Boehringer Mannheim Biochemicals (Indianapolis, IN). Plasmid pBR322 was obtained from Promega Biotec and pBluescript II SK (+/-), and VCS-M13 helper phage were from Stratagene. DNA primers were purchased from Stratagene, Promega Biotec, and BRL Life Technologies. Sequenase 2.0 sequencing kit and Ultra-Pure sequencing reagents were obtained from United States Biochemical Corporation.

Other reagents, kits, and buffers used are listed categorically by method in Table 2:

Table 2. Reagent and buffer components.

Reagents and buffers	Components
A. Plasmid DNA isolation and purification	
Luria broth (LB)	10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl. Adjust to pH 7.0. Plates: reduce to 1 g/l NaCl, pH to 7.4, add 2% agar.
50 mM TE buffer (pH 8.0)	6.1 g/l Trizma base, 18.4 g/l Disodium ethylene-diamine-tetraacetic acid (Na_2EDTA), pH 8.0.
3 M Potassium acetate (KAc)	For 100 ml: Add 29.4 g KAc to 50 ml d-H_2O and adjust pH to 4.8 with glacial acetic acid and bring volume to 100 ml with d-H_2O.
RNAse stock	10 mg/ml RNAse A in d-H_2O. Boil 15 min to deactivate DNAses. Cool slowly and store at -20°C.
Sephacryl S-1000 buffer	10 mM Tris-phosphate (pH 8.0), 1 M NaCl, 1 mM Na_2EDTA

Table 2. Reagent and buffer components, continued.

Reagents and buffers	Components
DEAE Rinse buffer	0.1 M NaCl, 0.1 mM Na_2EDTA, 10 mM Tris (pH 8.0)
DEAE Elution buffer	1 M NaCl, 0.1 mM Na_2EDTA, 10 mM Tris (pH 8.0)
X-GAL	5-Bromo-4-chloro-3-inolyl-ß-D-galactoside dissolved in dimethyl formamide (20 mg/ml) stored at -20^0 C. Add 80 µg/ml LB agar or 40µl stock added to LB plate surface before plating cells.
IPTG	Isopropyl-1-thio-ß-D-galactoside dissolved in st-d-H_2O (0.1M) added to LB agar at 20 mM or 4 µl to LB surface. Store stock at -20^0C.
SuperBroth (SB)	35 g/l Tryptone, 20 g/l yeast extract, 5 g/l NaCl, pH 7.5.
B. Electrophoresis buffers and solutions	
10 x TAE	48.4 g/l Trizma base, 16 ml/l glacial acetic acid, 4.1 g/l anhyd. sodium acetate, 3.72 g/l Na_2EDTA. pH 7.8.
10 x TBE	118 g/l Trizma base, 55 g/l boric acid, 6.72 g/l Na_2EDTA, pH 8.0.
10 x MOPS buffer (295) [p4.9]	1.8 g 4-morpholinepropanesulfonic acid plus 800 ml sterile Diethyl-pyrocarbonate treated (DEPC-Tx) d-H_2O, adjust pH to 7.0. Add 16.6 ml 3 M NaAc (DEPC-Tx), 20.0 ml 0.5 M EDTA pH 8.0 (DEPC-Tx). Add DEPC-Tx water to 1 l. Filter sterilize and protect from light. Store at RT.
Ethidium bromide (EtBr)	1 mg/ml EtBr stock solution in water solutiondiluted to 1 ug/ml for staining. Store at 4^0C.
Sample loading buffer ("Sugelpot")	60% (w/v) Sucrose, 0.1% SDS, 5 mM Na_2EDTA, 1% bromophenol blue and Xylene cyanol

Table 2. Reagent and buffer components, continued.

Reagents and buffers	Components
30% Acrylamide stock	29g acrylamide, 1g N,N'-methylene-bisacrylamide, add d-H_2O to 100 mls. Filter (0.45 µm) and store at 4°C.
3% Ammonium persulfate (APS)	0.3g APS/10 ml water. Store at 4°C.
CHEF loading dye (Contour-Clamped Homogeneous Electrical Field = CHEF)	0.6% Low-M agarose in 1 X TAE plus 0.25% bromophenol blue
Formaldehyde sample loading buffer	50% glycerol, 1 mM Na_2EDTA pH 8.0 0.25% bromophenol blue, 0.25% xylene cyanol FF
C. French Pressure Cell DNA isolation	
$Li_{3.6}EDTA$ (pH 8.0)	For 500 mls: Mix 35.5 g free-acid EDTA and 10 g LiOH in 400 ml d-H_2O. Check pH and slowly add more LiOH until pH 8.0 . Add d-H_2O to 500 mls, autoclave, and store at RT.
A-1 buffer (pH 7.5)	1 M Sorbitol, 0.25 M $Li_{3.6}EDTA$, 20% glycerol, 0.5% Triton X-100
High salt buffer (HSB)	25 mM Tris-HCl pH 7.4, 100 mM NaCl, 2 mM Na_2EDTA
Low salt buffer (LSB)	1 mM Tris-HCl pH 7.4, 1 mM NaCl, 0.1 mM Na_2EDTA
Wilcockson reagent (Ethanolic Perchlorate Reagent, EPR) (301)	For 500 mls: dissolve 122.44 g anhydrous sodium perchlorate in 53.6 ml of pre-warmed, 60°C d-H_2O. Filter solution through glass fiber filter (Whatman GF/C) followed by 381 ml of pre-warmed, 60°C 100% ethanol. Store in hood, label as "WARNING: EXPLOSIVE", and handle with gloves and eye protection.
SEVAG	Chloroform: Iso-amyl alcohol (24:1)

Table 2. Reagent and buffer components, continued.

Reagents and buffers	Components
D. Spheroplast genomic and mitochondrial DNA isolation	
Activation solution (ActS)	0.1 M EDTA pH 9.0, 0.3 M 2-mercaptoethanol (2-ME). (Stock concentration of 2-ME is 14.3 M)
Digestion solution (DigS)	1.0 M sorbitol, 0.1 M Na_2EDTA pH 7.5, and Zymolyase 20T at 80 U/gram packed cells.
Genomic lysis solution (GLS)	0.15 M NaCl, 0.1 M Na_2EDTA pH 8.0
Osmotic lysis solution (OLS)	0.25 M sucrose, 1 mM Na_2EDTA, 5 mM Tris-HCl pH 7.5
Mitochondrial DNAse buffer (mtDB)	0.3 M sucrose, 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.15% bovine serum albumin (BSA)
Mitochondrial wash buffer (mtWB)	0.3 M sucrose, 20 mM Tris-HCl pH 7.5, 10 mM Na_2EDTA, 0.15% BSA
Mitochondrial gradient buffer (mtGB)	0.1 mM M-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 mM Na_2EDTA pH 7.0
Organelle lysis buffer	10 mM Tris-HCl pH 8.2, 10 mM Na_2EDTA, 100 mM Nacl
E. CHEF chromosomal embedment	
Agarose mix	1% SeaPlaque agarose in 0.125 M Na_2EDTA pH 7.5
SCE	1 M sorbitol, 0.1 M sodium citrate pH 5.8, 0.01 M Na_2EDTA
Solution I	Mix: 10 mls SCE, 0.5 ml 14.3 M 2-ME, and add 1 mg/gram cells of Zymolyase 20T
Solution II	0.45 M Na_2EDTA pH 9.0, 10 mM Tris-HCl (pH 8.0), 7.5% (v/v) 2-ME
Solution III	0.45 M EDTA pH 9.0, 10 mM Tris-HCl pH 8.0, 0.1% Sarkosyl, and 1 mg/ml proteinase K

Table 2. Reagent and buffer components, continued.

Reagents and buffers	Components
F. RNA isolation buffers	
DEPC-Tx d-H_2O	0.2 ml diethylpyrocarbonate mixed with 100 ml d-H_2O for 30 min. All RNA buffers were prepared with DEPC-Tx water and autoclaved.
Extraction buffer 1 (EB1)	6 M guanidine-HCl, 10 mM dithiothreitol (DTT), 0.1 M Tris-HCl pH 6.5, 1% Sarkosyl
Buffer 2 (B2)	4.5 M NaAc pH 5.5
Buffer 3 (B3)	6 M guanidine-HCl, 0.1 M Tris-HCl pH 7.0, 20 mM Na_2EDTA, 10 mM DTT
Buffer 4 (B4)	0.01 M Tris-HCl pH 7.4, 4 mM Na_2EDTA, 2 mM DTT
5.7 M CsCl	Dissolve 9.59 g CsCl in 10 ml 0.1 M Na_2EDTA and DEPC-Tx. Autoclave and replace vol lost with sterile DEPC-Tx d-H_2O.
G. Southern blot transfer and hybridization solutions	
Denaturing NaOH-NaCl	0.5 N NaOH, 1.5 M NaCl
Neutralizing Tris-NaCl	Pre-mix 0.5 M Tris-HCl to pH 7.1. Add NaCl to 3.0 M and adjust volume.
20 X SSC	3 M NaCl, 0.3 M sodium citrate
GeneScreen Plus post-blot wash solutions	Immerse blot in 0.4 N NaOH for 30-60s. Rinse in 0.2 M Tris-HCl pH 7.5, 2 X SSC for 5-15 min.
5 X SSPE	0.15 M NaCl, 5 mM sodium phosphate pH 7.5 (6.9 g/l sodium monobasic phosphate + 7.1 g/l sodium dibasic phosphate), 1 mM Na_2EDTA.
H. Nick translation	
10 X Nick translation buffer (NTB)	500 mM Tris-Hcl pH 7.5, 100 mM $MgSO_4$, 1 mM DTT, 500 µg/ml BSA

Table 2. Reagent and buffer components, continued.

Reagents and buffers	Components
10 x dNTPs	5 mM solution of three cold deoxynucleotide triphosphates for ^{32}P-dTTP label: 5 mM dGTP, 5 mM dATP, 5 mM dCTP in 0.05 M Tris-HCl pH 7.0. Store at -20^0 C.
DNAse I stock solution	1 mg/ml DNAse I (1300 K.U./ml) in 50% (v/v) glycerol, 0.5 x NTB, 0.015% 2-ME. Store at -20^0 C.
DNAse working solution	1 x 10^{-5} dilution in 1 x NTB, 0.06% 2-ME. (Approx. 1 x 10^{-2} K.U./ml)
H. Sequenase 2.0 kit components	
5 x Sequenase buffer	200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCL
Enzyme dilution buffer	10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA
Sequenase Modified T7	20 mM potassium phosphate pH 7.4, DNA Polymerase, 1 mM DTT, 0.1 mM Na_2EDTA, 50% glycerol (13,000 U/ml)
5 x dGTP Labeling mix	Deoxynucleotides, 7.5 µM each: dGTP, dTTP, dCTP
5 x dITP Labeling mix	Deoxynucleotides: 15 µM dITP, 7.5 µM dCTP and dTTP
ddN Termination mixes (N = A, G, C, or T)	80:8 µM ratio deoxyNTP:dideoxyNTP, 80 µM each of three other deoxytriphosphates in 50 mM NaCl
ddX Termination Mix (X= A,G,C, or T)	160:1.6 µM ratio dITP:ddGTP or 160:8 ratio of dITP:ddA,T,or CTP, 80 µM for dITP rxns) each dATP, dCTP, and dTTP, and 50 mM NaCl
Stop solution	95% formamide, 20 mM Na_2EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF

Table 2. Reagent and buffer components, continued

Reagents and buffers	Components
I. Sequencing gel solutions	
40% Acrylamide stock	380 g/l acrylamide, 20 g/l bis-acrylamide in d-H_2O. Filter through Whatmann 3MM, store at 4^0 C in dark bottle.
6% Gel mix	For 100 ml: 15 ml 40% acrylamide stock, 10 ml 10 x TBE, 50 g urea, and 35 ml d-H_2O. Degass under vacuum.
10% Ammonium persulfate (APS)	1 g APS in 10 ml d-H_2O. Made weekly and stored at 4^0 C.

Methods

Plasmid Preparation

The cloning of HpaII fragments from C. albicans by J. Cutler and H. Horn has been described (99). Briefly, C. albicans 9938 genomic DNA was digested with HpaII, ligated to ClaI digested, phosphatase treated pBR322, and used to transform $CaCl_2$-competent αDH5 E. coli. Plasmid DNA was isolated from ampicillin resistant transformants and digested with MspI to screen for candidal inserts on 1% TAE agarose gels. The plasmid with the largest insert (by MspI digestion criterion) was designated pHpaCa7 and analyzed by the following methods.

Plasmid Isolation and Purification. An alkaline lysis procedure (with minimal NaOH exposure time) was used for preparation of bacterial plasmids (295,302,303). Bacterial cultures (1 l LB) were grown (12-16 h, 160 rpm, 37^0 C) with appropriate antibiotics (ampicillin, 50 µg/ml;

and/or tetracycline, 12.5 µg/ml). Cells were harvested (7,000 x g, 20 min, 4^0 C), washed in 100 ml 50 mM TE pH 8.0, suspended in 15 ml 20% (w/w) sucrose in 50 mM TE pH 8.0, and frozen at -70^0 C for at least 1 h. Fresh lysozyme solution (3 ml TE plus 15 mg lysozyme) was mixed with the thawn cell suspension and incubated on ice for 45 min. Freshly prepared 1% SDS, 0.2 N NaOH suspension (36 ml) was mixed in and left on ice 10 min before reducing the pH to 5.5 by addition of 3 M potassium acetate pH 4.8 (approximately 12 ml). After 40 min on ice, the precipitate (ppt) was pelleted (7,000 x g, 20 min, 4^0 C) and the supernatant fluid poured through a Kimwipe to trap any additional ppt. An equal volume of RT isopropanol was mixed with the fluid and the ppt pelleted (7,000 x g, 20 min) after 15-30 min at RT. The original procedure was modified after the isopropanol ppt to include EPR extraction, RNAse and proteinase K treatment, and SEVAG extraction as described in yeast genomic isolation section, below. If further purification was done, plasmids were chromatographed over a Sephacryl S1000 column according to supplier recommendations. Fractions were monitored for absorbance at OD_{260nm}, pooled, precipitated (ppt) with an equal volume of isopropanol and re-ppt in ethanol (EtOH).

Electrophoresis of DNA. Agarose gel electrophoresis (0.6-2.0% w/v agarose in 1 x TAE buffer) was used to test purity and separate fragment lengths of prepared plasmid or genomic and mitochondrial DNA (described below). Samples (intact or digested with restriction endonucleases 1-3 h) were mixed with HSB (if sample volume was <10 µl total) and 0.5 volume Sugelpot, loaded into sample wells and electrophoresed at 60 V for 10 min, then at reduced voltage for 7 cm minigels (1 h, 4-6 V/cm) or

17-24 cm long gels (12-20 h, 2.5-5 V/cm). Lambda phage DNA (digested with HindIII) and ϕX174 phage DNA (HaeIII digested) were routinely included for reference sizes. Gels were stained ≥ 15 min with EtBr staining solution, destained in water ≥ 15 min, placed on a UV transilluminator (Ultra-Violet Products, Inc., San Gabriel, CA), and photographed using a Polaroid MP-4 Land Camera (Cambridge, MA) with Polaroid Type 57 film (ISO 3000, f=8, 3 sec) or Type 55 film (ISO 50, f = 4.5, 50 sec) and a UV and orange filter (Kodak 7728G15). Film and negatives (Type 55 film) were developed as recommended. Fluorescent rulers (Diversified Biotech, Newton Centre, MA) were photographed on the gel for measuring migration and blotting dimensions.

Restriction fragments of pHpaCa7 were sized on regular agarose gels (above), 2-4% NuSieve agarose gels, and 3.5 - 5% polyacrylamide gels in TBE buffer [(304) p.173]. 30% stock acrylamide (11.6 ml for 3.5%, 16.6 ml for 5% gel) was mixed with 2.1 ml 3% APS, 10 ml 10 x TBE, and sufficient d-H_2O for 100 ml, degassed under vacuum, and 30 µl TEMED (N,N,N',N',-tetramethylethylenediamine) added. The acrylamide solution was poured into 16 x 20 cm vertical gel apparatus (BRL Model V16-2) and allowed to polymerize 60 min. After a 1 h, 200 V gel pre-run, sample wells were washed with buffer, the samples loaded and separated at 1 - 8 V/cm. Gels were EtBr stained and photographed as described above.

Purification of DNA Fragments. Electrophoretic purification of DNA fragments was done by either DEAE-paper ion exchange Transblot Membrane capture or Geneclean. For DEAE capture, the electrophoresed samples were EtBr stained, illumined briefly with long wave UV to identify band location and to slice the gel just ahead of the band. A small lane-

width strip of DEAE ion exchange paper was inserted into the slit and the band electrophoresed into the paper (40-60 V for 15-30 min). After checking for complete transfer by UV illumination, the DNA-bound DEAE paper was washed three times in DEAE rinse buffer (100 - 500 µl, depending on paper volume) to remove agarose and the DNA fragment was eluted off in 5 x 200 µl washes with DEAE elution buffer. Fragments were concentrated by adding 2.5 vol EtOH and ppt at -20^0C for ≥30 min.

DNA samples purified with Geneclean were treated as per kit instructions. Regular agarose, NuSieve GTG, or SeaKem GTG TAE-agarose gels were loaded to provide separate, duplicate lanes for staining and sample recovery. Gel slices containing the fragment of interest were weighed and melted at 45-50^0 C in the presence of 2.5-3 vol 6 M NaI. Glassmilk suspension (5 µl for ≤5 µg DNA, plus 1 µl for each 0.5 µg above 5 µg) was added, incubated for 5 min on ice or RT and centrifuged 5-10 sec. The pellet was washed 3 times with -20^0 C NEW wash (NaCl/Tris/EDTA buffered EtOH), suspended in water or TE buffer, and placed at 45-50^0 C for 3 min to elute the DNA. Free Glassmilk was removed by centrifuging 5-10 sec and transfering the supernatant fluid to a fresh eppendorf tube. Samples purified by DEAE and Geneclean were usually checked visually on a minigel prior to quantification by UV spectrophotometry, $OD_{260\text{-}280nm}$ readings.

Cellular DNA Preparation

Insert fragments of pHpaCa7 were radiolabeled for hybridization against Southern blots of various Candida species, fungi, and other mammalian DNA samples. Three methods for isolation of high molecular weight DNA from various Candida species, Torulopsis glabrata, and S.

cerevisiae cells have been described (298) and include modifications of Neurospora crassa and S. cerevisiae procedures (240,241,289,305,306). A brief description of two of the three methods used in this work follows.

French Pressure Cell Method. The Ribi Sorvall Cell Fractionator was utilized instead of a French pressure cell for large volume DNA extractions (liter size or larger). Mid-stationary yeast phase or mycelial phase cultures were washed in sterile d-H_2O and suspended in 3 vol A-1 buffer prior to passage through a pre-cooled Ribi Fractionator chamber [frozen overnight (o/n) at -20°C, then refrigerated 2 h prior to use] at 35,000-38,000 psi. Immediately, powdered SDS to a final concentration of 1% (w/v) and proteinase K (50 µg/ml) or Pronase (Sigma protease Type V to 250 µg/ml) were mixed with the cell lysate. Incubation of the lysate at 48° C for 2 h was followed by 1 h ppt in 2.5 vol EtOH at -20° C. The sample was centrifuged (6,000 x g, 10 min), supernatant fluid poured off, air evaporated, and the pellet suspended in HSB and re-centrifuged to remove cell debris (12,000 x g, 10 min). The supernatant fluid was ppt with 2.5 vol EtOH at -20° C for 1 h or o/n. After pelleting (12,000 x g, 10 min) and suspension in HSB, the preparation was extracted with 6 vol EPR, incubated on ice for 30 min, and centrifuged (6,000 x g, 10 min). The pellet was washed with 100% cold EtOH, allowed to air dry, suspended in LSB and incubated with RNAse (Sigma Type 1A.S to 5-10 µg/ml sample) at 37° C for 2-3 h. The EPR extraction was repeated, the DNA pellet dissolved in LSB, and extracted with an equal volume of SEVAG (vigorous shaking 5 min, centrifuged 3800 x g for 15 min). SEVAG extraction was repeated until the interface was visibly free of ppt. The aqueous DNA samples were ppt by adding 0.1

vol 5 M LiCl and 2.5 vol EtOH (-20^0 C, at least 30 min), centrifuged (12,000 x g, 20 min) and suspended in LSB or TE for enzyme studies or HSB for longer storage (4^0 C or -70^0 C).

Spheroplast Extraction Method. Yeast cells grown to mid-log phase in GYEP or stationary phase in mLee medium were treated as previously described to produce spheroplasts (305). Harvested cells were washed in 0.1 M Na_2EDTA (pH 9.0) and incubated with ActS (2 ml/gram cells, where 1 g of packed cell wet weight equals approx. 1 x 10^{10} cells) for 15 min before suspension in DigS (2ml/g) for 30 - 60 min at 37^0 C with gentle agitation. The spheroplasts were centrifuged gently at 2000 x g, 5 min and washed in 1.0 M sorbitol, 0.1 M EDTA pH 7.5. The cells were lysed in GLS, SDS and proteinase K added as described above with subsequent extraction steps. Spheroplast DNA extraction was also used for C. albicans cultures grown in the presence of demethylating agents (Appendix A) and radioactive phosphoric acid (Appendix B).

Mitochondrial DNA Isolation. A method for obtaining nuclear-free mitochondrial C. albicans DNA (mtDNA) was used (238). Spheroplasts of log-phase cells were prepared and centrifuged (2000g, 5 min) maintaining ice-cold conditions. The cells were washed twice in 0.9 M sorbitol, 0.1 M Na_2EDTA pH 7.5 prior to dilution in OLS. Several strokes (3-5) of a Dounce homogenizer were used to disrupt the cells and the suspension was left on ice 30 min prior to re-adjusting the pH to 7.5 with 10 N NaOH. The suspension was centrifuged (3000g, 5 min), the supernatant fluid saved and combined with supernatant fluid from a second homogenization of the pellet in OLS. The supernatant fluid was centrifuged twice

(1,500g, 10 min) to remove heavy debris. Mitochondria were pelleted at 15,000g for 15 min, suspended in mtDB with DNAse I (25 µg/ml) and incubated on ice for 30 min. The mitochondrial suspension was diluted three fold with mtWB, pelleted (12,000g, 30 min) and washed twice in mtWB. Mitochondria were banded in 25-50% (w/v) linear sucrose gradients in mtGB (centrifuged 90 min at 21,000 rpm, SW41Ti rotor, OTD65B Sorvall Ultracentrifuge). Bands were removed and diluted with one vol 25% (w/v) sucrose (in mtGB) and the mitochondria pelleted at 35,000 rpm for 30 min (SW41Ti rotor). The sucrose gradient was repeated and organelles lysed by suspending in OLS with 2% sarkosyl for 30 min. on ice. DNA isolation procedures, beginning with EPR extraction as described above, were used to prepare the final mtDNA. Appendix C describes methods tested in attempts to isolate purified nuclear DNA from C. albicans.

Mammalian DNA Isolation. Human peripheral leukocytes and mouse spleen lymphocytes were prepared as previously described (99). Briefly, heparinized human blood (normal whole blood obtained by venipuncture from Glee) was adjusted to 3% dextran (T250, Pharmacia Inc., Piscataway, NJ) for sedimentation of erythrocytes and separation of a leukocyte enriched fraction (307). The human lymphocytes were centrifuged (1,500 rpm IEC tabletop centrifuge, 30 min) and suspended in 10 mM Tris-HCl (pH 8.2), 10 mM Na_2EDTA, 100 mM NaCl. Lymphocytes from two adult male mice were obtained by washing out unattached cells from needle punctured (18-gauge) spleens with Tris-buffered saline, 10 mM Na_2EDTA. Both leukocyte suspensions were incubated with 100 µg/ml proteinase K and 2% sarkosyl for 3 h at 50^0 C before treating with EPR and subsequent DNA extraction steps, as above.

Chromosomal Preparation. The method of Carle and Olson was followed (269) to prepare cells with the exception that late-log cells C. albicans (GYEP) and S. cerevisiae (YPD), or mLee-grown yeast or hyphal phase cultures of C. albicans were used. Briefly, 100 ml cultures were washed twice in 50 mM Na_2EDTA pH 7.5 and cells suspended in 3.25 ml of the same solution. Five ml of 1% low-melting agarose (made in 0.125 M EDTA pH 7.5) was mixed with 3.0 ml cell suspension (approx. 2-4 x 10^9 cells/ml) and 1 ml Solution I. The mixture was poured into a 6 cm petri dish and allowed to harden. The dish was overlaid with 5 ml Solution II and incubated o/n at 37^0 C in a sealed plastic bag. The overlay solution was replaced with 5 ml Solution III, resealed and set at 50^0 C o/n. Plates were stored at 4^0 C with a final replacement overlay of 0.5 M Na_2EDTA pH 9.0.

Well-sized slices from the embedment plates were loaded in the 1% agarose gel (in 0.5 x TBE buffer) and sealed with CHEF loading dye. The gel was placed into the electrophoresis chamber (Owl Scientific) and pre-iced 0.5 x TBE buffer was added. The DNAstar Plus Programmer (Version 65, DNAstar, Inc, Madison, WI) controlled the electrophoretic parameters of 48-80 h and 400 sec switching time at a constant 100 volts. Buffer was circulated (Masterflex Circulator by Cole Parmer Instrument, Co.) and cooled to $6-10^0$ C (Polyscience Corp. refrigeration unit, Niles, Il). Final electrophoretic buffer temperature ranged from $10-14^0$ C. Gels were stained in EtBr (1 µg/ml) 30 min and destained 1 h prior to photographing.

Hybridization Analysis

Southern Blots and Radiolabeling. TAE agarose gels were routinely placed in 0.25 N HCl for 15 min to depurinate the DNA fragments (296), denatured in 0.4 N NaOH - 0.6 M NaCl for 30-45 min, rinsed in water, neutralized in 0.5 M Tris - 1.5 M NaCl pH 7.5 for 30 min, and capillary blotted to GeneScreen Plus nylon-based membrane. After 4 - 24 h of transfer, the blots were treated in 0.4 N NaOH for 30 - 60 sec and rinsed in an excess of 0.2 M Tris-HCl pH 7.5, 2 x SSC, and allowed to air dry.

Preparation of ^{32}P labeled DNA was by the nick translation method described by Rigby, et.al. (308) with modifications (240) as follows: 0.5 µg DNA was mixed with 10 µl 10 x NTB, 10 µl 10 x dNTPs, 1 µl 3% 2-ME, 1 µl 1 M DTT, 10 µl DNAse working solution, 2 µl DNA pol I, 25 µl α-^{32}P dTTP or dCTP (3,000 Ci/mM), and sufficient d-H_2O to total 100 µl. The mixture was incubated at 12-14^0 C for 2 h, ppt with 2.5 vol cold EtOH, and set at -20^0 C (1 h or o/n) or -70^0 C (15-30 min). The labeled DNA fragments were centrifuged (10-15 min in microfuge) and the supernatant fluid saved. The pellet was centrifuged 2 min with 50 µl fresh cold 70% EtOH (supernatant fluid combined with the first), and air-dried before suspension in 200 µl HSB. The relative % incorporation was determined by Cerenkov scintillation counting of 1 µl of supernatant fluid (containing unincorporated label) and 1 µl of resuspended probe [(probe cpm/(probe + sup cpms) x 100]. For some experiments, labeling of DNA was done by Probe-eze (random hexanucleotide primer kit) or the BRL Nick Translation Kit. All methods produced probes within the specific activity range of 1 - 4 x 10^8 cpm/µg DNA.

Southern hybridization of the nylon based membrane, Genescreen Plus, was according to manufacturer instructions (NEN catalog no. NEF-976) with the following modifications. Prehybridization of blots was carried out in 5 x SSPE solution (1 ml/10 cm^2 membrane) for 15 min - 2 h at 65^0 C prior to addition of probe. Incubation of blots with added probe (2 x 10^7 cpm/bag) continued at 65^0 C with constant agitation for 4-30 h. Blots were washed 5 x 100 ml (5 min at 65^0 C) in 2 x SSC, 1% SDS with agitation and followed by a stringent wash in 0.1 x SSC, 0.1% SDS for 30 min at 48^0 C. Probe-hybridized membranes were placed on 3MM paper, labeled with radioactive ink, covered with plastic wrap and sandwiched next to Kodak X-Omat XAR-5 film for RT exposure or with addition of a calcium-tungstate-phosphor intensifying screen (Cronex Lightning-Plus, NEN Research Products, Dupont) at -70^0 C for enhanced band development. Autoradiograph film was processed using Kodak GBX developer and fixer.

Dot Blots. Actin gene and pHpaCa7 large piece inserts were Geneclean purified for use in dot blot analysis. Genomic C. albicans DNA was pre-digested with EcoRI and SEVAG extracted before use as target DNA. All DNA samples for use in the dot blot screening, whether genomic or purified fragments, were quantified using UV spectrophotometry after isolation or digestion to establish stock solutions with known concentrations. The DNA samples were denatured in 0.4 N NaOH, 10 mM EDTA by heating to 100^0 C for 10 min, neutralized by addition of 1 vol cold 2 M ammonium acetate, pH 7.0, and adding SBB to make working stock solutions of denatured target DNAs: 1 pg/µl and 10 pg/µl for standard curve DNA, and 0.5 µg/µl genomic target DNA. Sheared salmon sperm DNA was denatured and added (0.5 µg/well) as a carrier with 100 µl SBB per

microtiter plate well. The denatured insert and denatured genomic DNA were added in varying amounts (added volumes did not exceed 30 µl) to microtiter wells. Pre-wetted Genescreen Plus was assembled in the Bio-Dot Microfiltration apparatus (Bio-Rad) with gentle vacuum while securing the top. Samples were added without suction and allowed to sit 30 min before gentle suction was applied. Wells were washed with 200 µl 0.4 N NaOH, brief vacuum applied, and the blot treated as described above for Southern blots.

RNA Extraction and Northern Blots. Yeast phase and mycelial phase total RNA was extracted using a guanidine-HCl protocol [(295) p4.2.1-2, (309). C. albicans 9938 and Ca 1 cells were grown in GYEP and mLee (for morphogenesis induction), harvested by centrifugation or vacuum filtration for mycelia (1.2 µm Type RA Millipore filter) and suspended in EB1. The Ribi Fractionator was washed extensively in DEPC-Tx water and sterile DEPC-Tx water prior to breaking the yeast or hyphae. Broken cell samples were centrifuged (10,000 rpm, GSA, 10 min), pellets re-extracted with EB1 and centrifuged, and 0.04 vol B2 added to the pooled supernatant fluids. Ethanol (0.75 vol) was added and the samples ppt o/n at -20^0 C. After centrifuging (4,100 x g, 1 hr, 0^0 C), the air-dried pellets were suspended in minimal vol B3 and drawn four times through a 20 ga. needle before layering onto 1-1.5 ml of 5.7 M CsCl in siliconized, autoclaved SW50.1 polyallomer tubes. Samples were centrifuged 22 h, 36,000 rpm at 20^0 C to pellet the RNA through the CsCl layer. The pellet was suspended in B4 by heating to 60^0 C for 10 min and vortexing briefly. A portion of the yeast RNA was twice

chromatographed over oligo(dT) cellulose [(295) p.4.5.1-3] for enrichment of the messenger RNA (mRNA) population.

Alternatively, total RNA was prepared by an acidic phenol extraction method [(296) p.7.10-11] to allow isolation of small RNAs (5S and tRNAs) that may not sediment through the CsCl step gradient. A portion of the 9938 yeast RNA sample in B3, above, was ppt in 2.5 vol EtOH and suspended in 2 ml of 10 mM EDTA pH 8.0, 0.5% SDS plus 2 ml of 0.1 M sodium acetate pH 5.2, 10 mM EDTA pH 8.0 before organic extraction and twice ppt in EtOH.

Two aliquots of 9938 yeast RNA, one from each extraction method (CsCl and acidic-phenol), were treated with RQ1-DNAse (Promega) as described [(295) p4.1.4]. Spectrophotometric readings at OD_{260nm} were used to quantify total RNA yields and the DNA Dipstick kit was used to estimate mRNA concentration. RNA samples were aliquoted and stored as ethanol ppt at -70^0 C until electrophoresis or dot blot hybridization.

Yeast and mycelial (CsCl prepared) RNA was electrophoresed in formaldehyde agarose gels and capillary transferred to nitrocellulose as described [(295) p4.9.1, (296) p7.46-48]. Agarose (2.1 g + 152 ml water) at 60^0 C was combined with 17.5 ml 10 x MOPS buffer and 5.25 ml 37% formaldehyde and allowed to solidify for 30-45 min at RT. Formamide was deionized with BioRad AG 501-X8(D) (5 g resin/100ml formamide, stirred 1 hr, to a final pH of 7.1) and stored under nitrogen at -70^0 C. RNA in water (0.5-20 µg in maximum vol of 11.25 µl) was mixed with 10 µl 10 x MOPS buffer, 8.75 µl 37% formaldehyde, and 25 µl deionized formamide. Samples were heated 15 min at 55^0 C, mixed with 10 µl of formaldehyde loading buffer, and electrophoresed at 3-4 V/cm in an 18 cm

gel for 4 h. Gels with three-fold higher formaldehyde concentration (3.3% instead of 1.1% v/v formaldehyde) were also used in 6-8 h electrophoretic runs. Duplicate lanes were excised for staining or blotting to nitrocellulose. All nitrocellulose blots were baked (80° C vacuum oven, 2 h) before hybridization [(296) p7.52]. The blots were prehybridized at 42^0 C in 50% formamide, 5 x SSPE, 2 x Denhardt reagent, and 0.1% SDS for 1-2 h; then labeled probe (0.1 µg of > 2 x 10^8 cpm/µg DNA) added for hybridization (16-24 h). Blots were washed 20 min in RT 1xSSC + 0.1% SDS followed by 3 x 20 min, 65 - 68^0 C in 0.2xSSC + 0.1% SDS before autoradiography.

Various amounts of total RNA (both CsCl and acid phenol methods) and mRNA were prepared for dot blot application [(296) p7.54]. RNA in 10 µl water was mixed with 20 µl formamide, 7µl 37% formaldehyde, and 2 µl 20xSSC, heated 15 min at 68^0 C, and placed on iced. Nitrocellulose was presoaked for 1 h in 20xSSC, placed in the 0.1 N NaOH pre-cleaned Bio-Dot manifold, and the wells washed and refilled with 10xSSC prior to sample application. Samples were added, gentle suction applied, wells washed with 2 x 1 ml 10xSSC, and suction continued for 5 min. The membrane was air-dried, baked, prehybridized, and hybridized as described above except that 100 µg/ml denatured salmon sperm DNA was added to the formamide hybridization step.

pHpaCa7 Sequence Analysis

Subcloning. The pBluescript SK II(+) phagemid and pHpaCa7 were digested with PstI and electrophoresed to check for complete cutting. Twenty µl of phagemid (2 µg) digest was mixed with 10 µl 1 M Tris pH

9.0, 0.5 µl of 5 M NaCl, and 70 µl d-H_2O. Calf intestinal alkaline phosphatase (CIP) was added (0.5 µl of 20 U/µl stock) and the solution incubated at 37^0 C for 30 min. A second aliquot of CIP was added and incubated for 30 min at 50^0 C to decrease vector background (302). Na_2EDTA was added to the vector mixture to 5 mM and heated (75^0 C, 10 min). PstI-CIP treated phagemid and digested pHpaCa7 were extracted with an equal vol of Tris-buffered phenol:chloroform (296) and 0.1 vol 3 M sodium acetate pH 7.0 was added before 2 vols EtOH to ppt the DNAs (-70^0 C, ≥30 min). Pellets were washed with 70% EtOH, dried, and suspended in d-H_2O. A 1:1 ratio of phagemid:pHpaCa7 (approx. 0.2 µg DNA each) was ligated in 10 µl of 1 mM ATP, 1 x T4 ligation buffer (50 mM Tris-HCl pH 7.5, 7 mM $MgCl_2$, 1 mM DTT), 1.5 U T4 ligase, for 4-8 h at 14^0 C. Half of the ligation mix was used to transform E. coli XL1-Blue 2 competent cells prepared by the $CaCl_2$ method (295). XL1-Blue cells were harvested from a 50 ml LB log phase culture (OD_{550nm} 0.4), centrifuged (SS34, 10K, 10 min) and incubated on ice in 25 ml 0.1 M $CaCl_2$ for 25 min. Pelleted cells (SS34, 5K, 15 min) were suspended in 0.5 ml 0.1 M $CaCl_2$ and 80 - 100 µl aliquots were incubated with ligation mixtures for 1 h on ice before being heat shocked (42^0 C, 2 min). The transformation mix was diluted into 2 ml pre-warmed LB and incubated 2 h at 37^0 C prior to plating on selective LB (amp, tet, X-GAL, and IPTG) plates. Plasmid DNA from white bacterial colonies was prepared and screened for the presence of HpaCa7 PstI inserts.

J. Cutler, T. Kanbe, and L. LaTray subcloned agarose purified MspI fragments of pHpaCa7 into the ClaI site of pBluescript II SK(-). Some of their subclones were used for sequencing in this project.

Dideoxy Sequencing of Plasmids. Sequencing of pHpaCa7 and pBluescript subclones was done using Sequenase (Version 2.0) and associated protocols. Template plasmid DNA (2-4 µg), prepared with minimal NaOH lysis, was denatured in 0.2 N NaOH, 0.2 M EDTA for 30 min at 37° C, neutralized with 0.1 vol 3 M sodium acetate (pH 4.5 - 5.5), and ppt with 4 vol cold EtOH for 15 min at -70° C or longer at -20° C. The centrifuged pellet was washed in 70% EtOH, dried, suspended in 7µl water, mixed with 2 µl Reaction Buffer, 1 µl appropriate primer (0.5 pmol), heated to 65° C for 2 min, and allowed to gradually cool in a heat block. The annealed template-primer reaction was mixed with 1µl 0.1 M DTT, 2µl labeling nucleotide mix (dGTP or dITP, usually diluted 1:5 with d-H_2O), 0.5µl [α-^{35}S] dATP, and 3 U Sequenase (2µl of 1:8 dilution in Enzyme Dilution Buffer). The labeling reaction was incubated 5 min at RT, 3.5µl transferred to 4 prewarmed termination tubes (37° C) containing 2.5µl of G, A, T, or C termination mix, and returned to 37° C for 5 min before adding 4µl of Stop Solution. Two pBR322 primers [EcoRI clockwise (cw) primer, 5'-GTATCACGAGGCCCTT-3'; and HindIII counterclockwise (ccw) primer, 5'-GCAATTTAACTGTGAT-3'] were used to obtain sequence information from pHpaCa7, and the M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-AACAGCTATGACCATG-3') primers were used for pBluescript annealing. The dGTP or dITP labeling mix was diluted 1:10 for obtaining sequence near primers (electrophoresed on 8% acrylamide-urea gels) and used undiluted for reading greater than 250 nucleotides from the primer.

The shorter glass plate of each sequencing set (BRL) was pre-treated with RAIN-X (310) to reduce gel adherence. RAIN-X liquid was applied

with a dampened cloth (Kimwipe) in circular motion, allowed to dry to a haze, and reapplied. After complete drying, the haze was removed with a water-moistened cloth and the plate washed with dishsoap. The RAIN-X treatment lasted for 8 - 10 gel runs.

Both glass plates of a set were prepared by handwashing in Bon-Ami cleanser, dishsoap, rinsing with tap and distilled water, and a final 70% EtOH rinse. Standard 6% polyacrylamide/urea mix was mixed with 1 ml 10% APS and 10 - 20 µl TEMED and immediately poured into 0.4 mm spacer gels. After 40 - 60 min polymerizing, gels were pre- electrophoresed (1300-1600 V, 30 min, BRL Model S2 System) before loading the heated sequencing reactions (75 - 80^0 C, 2 min). Sequencing gels were run for 2.5 - 12 h (short or long sequence information) with a 1 x TBE buffer change for gels longer than 6 h. Sequencing gels were fixed in 3 liters of 5% methanol/5% acetic acid (15 - 30 min), transferred to Whatman 3MM paper and vacuum dried at 80^0 C for 1 h (BioRad Model 583 Gel Dryer), and exposed 2 - 3 days with Kodak X-OMAT AR film.

VCS-M13 Rescue of ssDNA Templates. Standard procedures for phage amplification, titering, and storage [(296) p4.21-23] were followed for VCS-M13 (Stratagene) helper phage rescue of pBluescript II SK (±) ssDNA templates for sequencing. Briefly, phage was added to a 10 ml LB culture XL1-Blue E.coli (OD_{600nm}0.3) at a multiplicity of infection (MOI) of 20:1, grown 30 min at 37^0 C, kanamycin (25 µg/ml) added, and the cells incubated 8 h. The culture was heated (65^0 C, 15 min) and cell debris pelleted. Phage was either polyethylene glycol (PEG) ppt from the supernatant fluid for frozen storage or titered onto XL1-Blue cells. For rescue, a pBluescript (± subclone insert) harboring, XL1-Blue

culture was grown o/n and inoculated into 3 ml SuperBroth (SB) to give an OD_{600nm}=0.1. VCS-M13 phage were added during log phase (at MOI of 20:1) and the culture incubated another 8 h. Cell debris was pelleted (12,000 x g, 10 min) and 1.2 ml supernatant fluid transferred to a microfuge tube. Phage was ppt with 300 µl of PEG solution (20% PEG, 3.5 M ammonium acetate) at RT for 15 min and centrifuged (12,000 x g, 20 min). The pellet was suspended in 300 µl 10 mM TE (pH 8.0), an equal volume of Tris-buffered phenol/chloroform was added, and the layers vortexed 1 min. Extraction of the aqueous phase was repeated until the interface was clear and a final chloroform extraction was done. An equal vol of 7.5 M ammonium acetate (pH 7.5) was added to the final aqueous phase, and mixed with 2 vol EtOH to ppt ssDNA on ice for 15 min, and centrifuged 20 min. The pellet was washed with 80% EtOH, dried, and suspended in 20 µl 10 mM TE pH 8.0. Five µl (0.8 - 1.0 µg DNA) of the final ssDNA suspension (from 3 ml SB culture) was used as template DNA for sequencing as described above.

Sequence Analysis. Sequencing reactions (dGTP and dITP) were electrophoresed for short and long gel runs from each primer-template combination. The autoradiographs were read manually and the data entered into Asci II files using the Align program (Version 1.0, Scientific & Educational Software, State Line, PA). Data files were compared to determine the overlap of separate readings from long and short electrophoresis runs and to indicate any base discrepancies for duplicate electrophoretic runs of the same primed sequences. Films were rechecked to confirm or correct the differences or represent ambiguities by N's. Candida nucleotide information was used to query GenBank files

by FastA sequence similarity search (311) and was analyzed for the presence of open reading frames by Protylze predictor program (Version 3.0, Scientific and & Educational Software, State Line, PA).

RESULTS

DNA Extraction from Candida albicans

Isolation of high quality fungal DNA requires mechanical or biochemical disruption of the cell wall with limited shearing of the nucleic acids. Two methods, Ribi Cell fractionation and spheroplast extraction (reported in reference (298)), were found to have different experimental advantages though were equivalent at providing quality DNA from C. albicans. The autolysis DNA extraction method (298) was not used here.

Extracted DNA Quality

DNA extracted from C. albicans isolates using either Ribi Cell fractionation or the spheroplast method was of high molecular weight and readily cleaved by restriction enzymes. Uncut and digested DNA samples from six strains are shown in Figure 1. For both methods, the uncut sample lanes in panel A and C indicate that most Candida fragments were larger than the 23.1 kbp DNA standard fragment from Lambda phage cut with HindIII (lane 1 in panel A and B, lanes 3 and 10 in panel C). Electrophoresis of the uncut samples on 0.5 or 0.7% agarose gels, containing a Lambda DNA x SalI standard in which 32.7 kbp is the largest marker piece, displayed sizes larger than 30 kbp (data not shown).

The digested DNA sample lanes in Figure 1 have EcoRI (G↓AATTC, panel C) and MspI (C↓CGG, panel B) bands which extend into the range of 23

kbp, but are generally shorter than the uncut samples. Fragment patterns were not altered by increasing enzyme amounts or prolonging the incubation, indicating that the digests were complete (data not shown). DNA samples, prepared by either method from various Candida isolates, were consistently susceptible to enzymatic cleavage. Though the overall quantity of DNA was not of primary interest, under the conditions described in the methods, approximately 2 µg DNA was obtained from 1 x 10^7 cells.

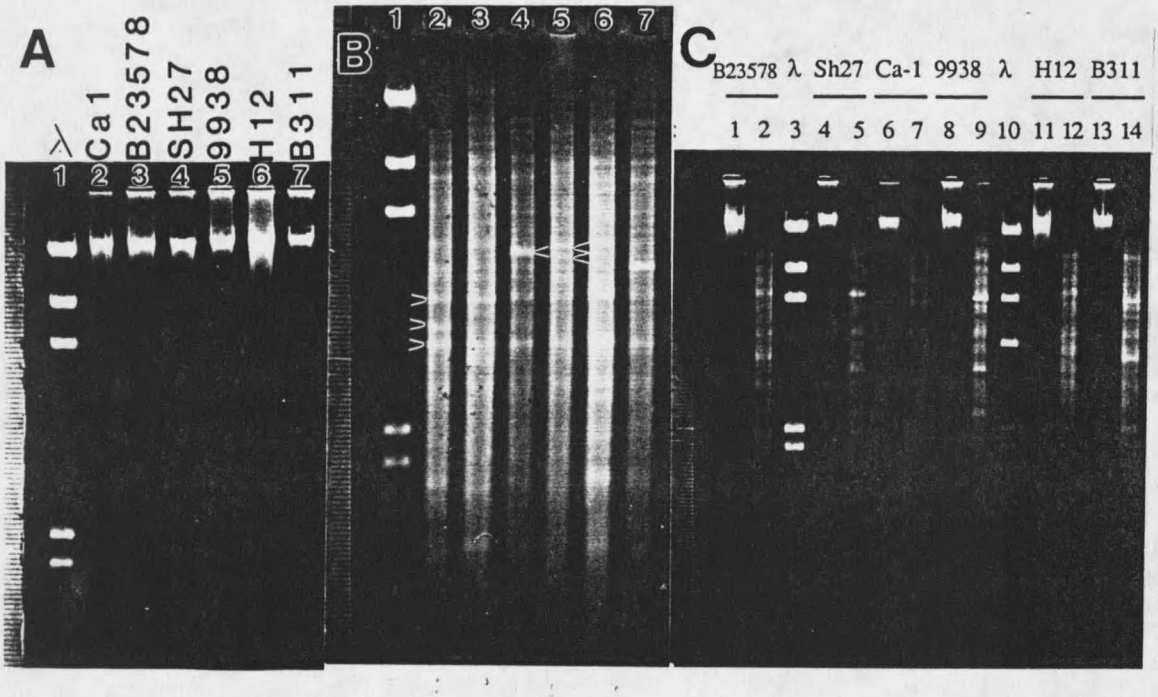


Figure 1. DNA extracted from C. albicans. Genomic DNA from six different isolates were extracted by two methods. Samples were electrophoresed in 1% TAE agarose gels, EtBr stained, and photographed. Panel A and B: Ribi Cell fractionation method. Lane 1 - Lambda DNA x HindIII standards; lane 2 to 7 - labels of uncut DNAs in panel A correspond to the same MspI-digested samples in panel B. Arrowhead marked EtBr intense bands are described in the text. Panel C: Spheroplast lysis method. The two lanes for each isolate represent uncut (left) and EcoRI digested DNA (right). Lanes labeled Lambda are DNA marker lanes containing fragments produced by HindIII digestion (23.13, 9.42, 6.68, 4.37, 2.32, and 2.03 kilobase pair [kbp]).

The restriction patterns of various isolates were generally similar for any particular enzyme, but some specific band differences were observed. For example, in the MspI digests of Figure 1, panel B, an intense band at 5.4 kbp (GEL program estimate (307)) was seen in isolate Sh27 DNA (lane 4, arrowhead), compared with bands at 5.6 and 5.3 kbp for 9938 (lane 5, arrowheads). For all C. albicans DNA samples tested, MspI digestion produced a three band group at approximately 4.2, 3.6, and 3.4 kbp (arrows in lane 1, GEL program estimates). The staining intensities of that group and larger bands indicate that the fragments may be repetitive. The putative repeated sequences are not from Candida rDNA repeat units because similar MspI blots probed with radiolabeled N. crassa rDNA showed hybridization sizes no larger than 1.5 kbp (J. Cutler, unpublished data, GEL program estimates). The number of MspI fragments in the higher molecular weight ranges suggests long A-T rich regions devoid of unmethylated CCGG sites or CCGG sites with the first cytosine modified by methylation in C. albicans genomic DNA. MspI will not digest ^{m}CCGG sites (312). (Appendix B includes estimates of MspI fragment size distribution.)

Comparison of Extraction Methods

Experimental application of the two extraction methods displayed important differences. The efficiency of spheroplast production with Zymolyase 20T varied with growth phase, cell morphology, and culture medium tested. Two of five isolates of Candida grown in GYEP showed

decreased spheroplast formation with entry into stationary phase (Figure 2). Isolate B311 showed the largest reduction in percent spheroplasts formed, from >95% to <50% going from Klett 400 to 500 growth (ie. stationary phase). Some strains displayed an overall lower susceptibility to Zymolyase digestion. For example, only 80% spheroplasting efficiency was obtained with strain 9938 grown in GYEP even for log-phase (Klett 400) cells. (Appendix D shows the relationship for Klett values to C. albicans cell density.)

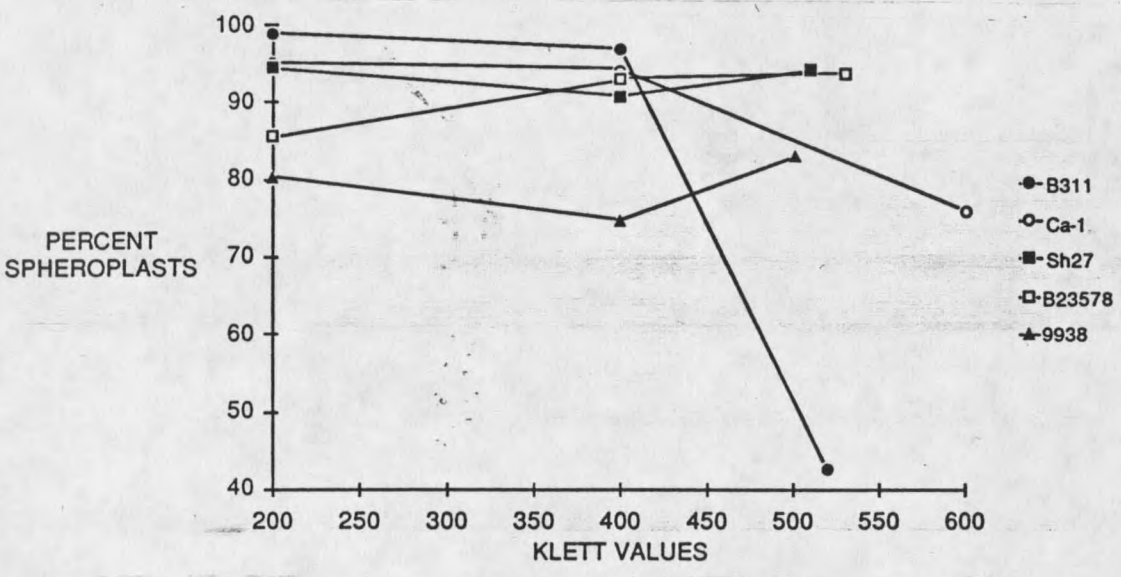


Figure 2. Efficiency of spheroplast induction in GYEP. The efficiency of spheroplast induction was assayed by diluting 10µl of the cell suspension into 1 ml of 1% (w/v) SDS. The intact cells were enumerated and compared with control dilutions made before the addition of Zymolyase 20T. Results of five strains of C. albicans sampled after 60 min of enzyme exposure are expressed as a percentage of the total population successfully spheroplasted. B23578 (□), Sh27 (■), Ca1 (○), 9938 (▲), and B311 (●) growth-curve analyses revealed that by Klett values of 500 or greater, each strain had entered early to mid-stationary phase.

This decreased susceptibility to spheroplasting with age has also been observed by others who have used cell wall digestive enzymes such as helicase and glusulase (from Helix pomatia (287)) to induce C. albicans spheroplasts (290,293). Practical application of the spheroplast method for cells grown in GYEP is limited by the number of culture strains that can monitored and harvested during log-phase for processing. Though each culture begins at Klett 100, differences in growth rates of individual strains complicate coordination of subsequent spheroplasting steps. Alternately, each culture may be harvested in log phase and cells placed in ice or frozen until all strains are ready to spheroplast. DNA extracted from iced cultures (2-3 h) was identical in quality to the samples in panel C, Figure 1 (data not shown).

In contrast, yeast cultures (strains 9938, B311, and Ca105), grown to stationary phase (48 h culture) in mLee medium, spheroplasted at 95 - 100% efficiency (data not shown). Thus, many yeast DNA samples could be prepared at one time from growth in mLee medium.

The spheroplast DNA method does not work well for C. albicans cells germinated in mLee medium. Three different isolates were germinated 2 - 3 hours in mLee temperature-shift induction (strains Ca1, Ca105, and CaOs produced true germ tubes) and incubated with Zymolyase 20T. Microscopic examination after 60 minutes of digestion revealed extensive cell lysis with only the ungerminated portion of the original population stable as spheroplasts (data not shown). Zymolyase 20T contains ß-1,3-glucanase, protease, and mannanase. The differing susceptibility of Candida isolates with respect to growth phase, form, and culture media

suggests osmotic or cell wall structural/chemical changes relevant to the enzymatic activities of Zymolyase.

The Ribi Cell fractionation method applied to mycelial or pseudohyphal form cells produced high quality DNA (Figure 17). With an available Ribi fractionator, this method is preferred (compared to the expense of Zymolyase) for processing large volume samples in any phase of growth; but not for processing many samples simultaneously due to rinsing and cold temperature requirements for the breakage chamber.

Analysis of Candida albicans Specific DNA

Plasmid pHpaCa7 was generated by Cutler and Horn (99) during attempts to clone the intense triplet bands described in Figure 1 which resulted from MspI (or HpaII) digests of Candida genomic DNA. Ligation of HpaII fragments into the ClaI site of pBR322 does not regenerate a ClaI or MspI/HpaII recognition sequence, eliminating recovery of the cloned fragment by either enzyme. In pBR322, the closest MspI sites flanking the ClaI insertion point are 438 bp 5', and 136 bp on the 3' side. In their experiment, MspI-digested plasmids were screened for C. albicans insert sizes on 1% TAE agarose, Figure 3. The largest insert observed was in pHpaCa7 (lane 7, arrow), which by GEL program estimate (307) was 3.58 ± 0.10 kbp for this gel. This insert was DEAE purified and used for the initial hybridization surveys and restriction mapping described below and reported in reference (99). All of the Southern blots done in this work were processed under high stringency wash conditions (high temperature, low salt) (295).

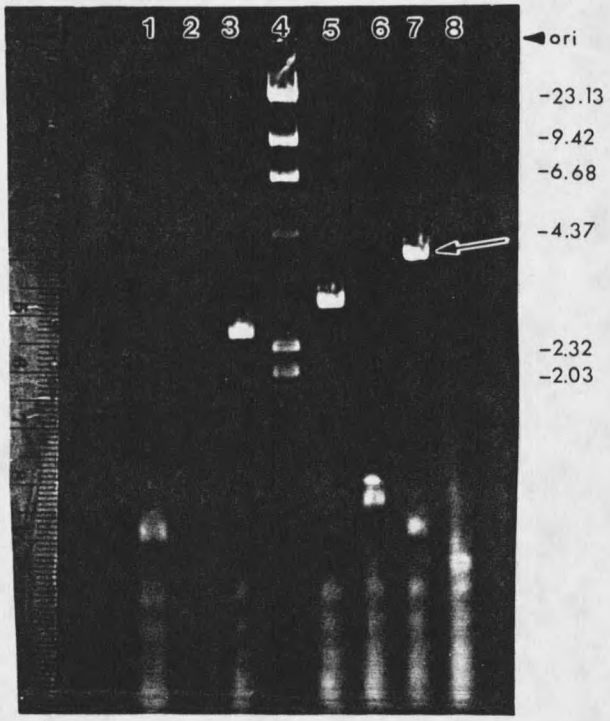


Figure 3. MspI digests of recombinant clones. Lanes 1-3 and 5-8 are miniprep plasmid DNAs digested with MspI and separated in 1% TAE agarose. Lane 4 - Lambda HindIII marker, Lane 7 - pHpaCa7 with an arrow marking the largest insert piece.

The insert was radiolabeled and used to probe Southern blots of seven Candida species, Gaeumanomyces graminis, Neurospora crassa, and human and mouse DNA. Figure 4 and 5 show the EtBr stained gels and corresponding autoradiograph images of probe hybridization. The only hybridization observed in Figure 4 in lanes 2, 3, 4, and 6 (panel B) were for C. albicans isolates. Figure 5 exhibits signals for C. stellatoidea ATCC 36232 in lane 1 and for C. albicans in lane 5 (panel B). No bands appeared in other fungal DNA lanes or in human or mouse DNA.

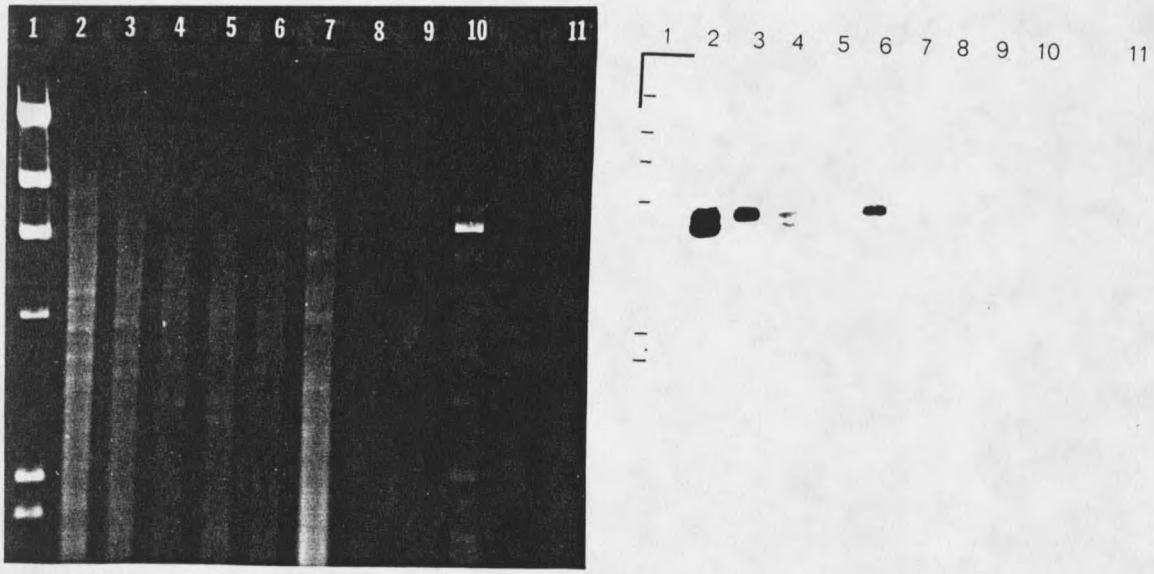


Figure 4. Gel and Southern blot of various fungi. Panel A: EtBr stained DNA fragment patterns of MspI-digested fungal DNA. Lane 1 - Lambda x HindIII marker, lanes 2, 3, 4, and 6 contain DNA from C. albicans 02, 23,63, and 13, respectively. Lanes 5 and 8 - C. tropicalis, lane 7 - C. guilliermondii, lanes 9 and 10- G. graminis vars. graminis and avenae, respectively, and lane 11- N. crassa. Panel B: Autoradiograph of Southern blot probed with the largest pHpaCa7 insert piece.

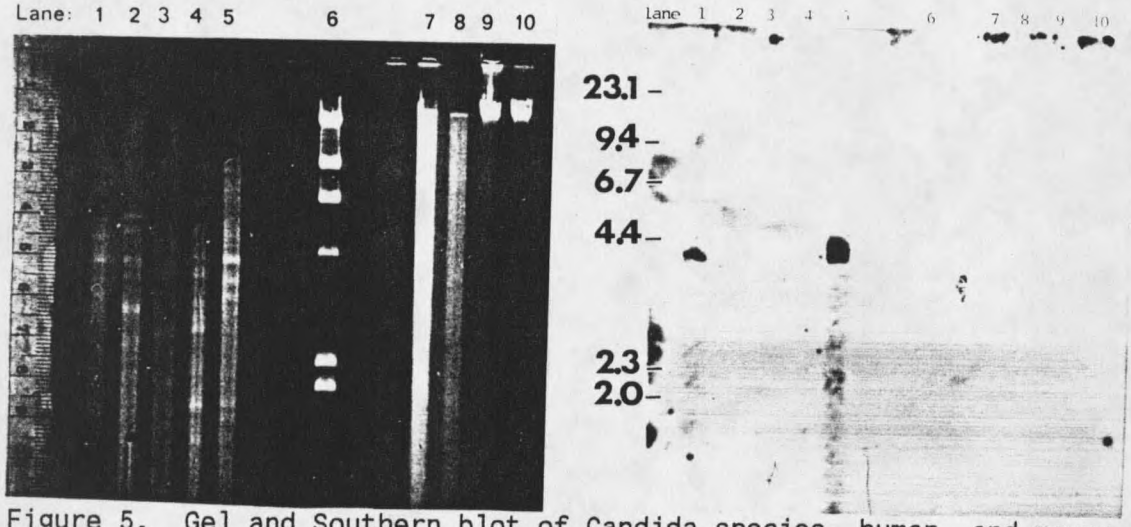


Figure 5. Gel and Southern blot of Candida species, human, and mouse DNA. Panel A: EtBr stained gel of MspI-digested Candida and mammalian DNA samples. Lanes 1 - 5 contain C. stellatoidea ATCC 36232, C. lusitaniae, C. krusei, C. parapsilosis, and C. albicans, respectively. Lane 7 - MspI cut mouse DNA, lane 8 - MspI cut human DNA, lane 9 - uncut mouse, and lane 10 - uncut human DNA. Panel B: Autoradiogram of Southern blot probed with labeled pHpaCa7 insert piece.

The hybridization bands seen for the C. albicans and C. stellatoidea samples in Figure 4 and 5 were not due to pBR322 sequences contained in the labeled probe. Similar Candida genomic blots probed with radiolabeled pBR322 indicated no cross-hybridization (data not shown).

The purified insert piece was incubated with 24 restriction enzymes (known to cut Candida genomic DNA), but only seven enzymes cut the insert. Fragments produced by single and double digests were sized on 1 - 4% agarose and 3.5 - 5% polyacrylamide gels (data not shown). Assuming that MspI-digested plasmid insert piece had the pBR322 flanks attached, the restriction enzyme information was combined with known enzyme sites of attached pBR322 flanks for a restriction map (Fig.6).

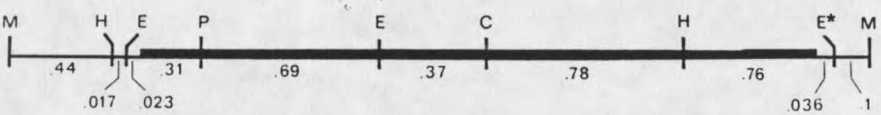


Figure 6. Restriction enzyme map of the pHpaCa7 insert piece. The fungal DNA HpaII fragment was cloned into the ClaI site of pBR322 and then retrieved from pHpaCa7 by MspI digestion leaving pBR322 sequences associated with the candidal DNA fragment. C. albicans DNA sequences are denoted by the thick line and pBR322 sequences by the thin line. Abbreviations: M - MspI, H - HaeIII, E - EcoRI, E* - EcoRI star site, P - PstI, C - ClaI, numbers refer to kilobase pairs of DNA. Multiple fragments from AluI, TaqI, and HincII digestion were also present but their locations were not mapped (99).

The Candida DNA portion of the above fragment was estimated at 2.9 kbp by subtracting the flanking 574 bp of pBR322. But, the sizes of hybridization bands in MspI-digested genomic C. albicans (Figure 4 and 5) was 3.8 to 4.2 kbp. Investigation of this apparent size difference led to finding additional C. albicans DNA pieces in the insert region of pHpaCa7, as described below.

Modified Restriction Map of pHpaCa7

MspI-digested plasmid pHpaCa7 yielded three fragments larger than similarly digested pBR322 (Figure 7, lanes 5 and 6). The three pieces were designated "L" for large, "M" for medium, and "S" for the smallest piece and are labeled with letters in Figure 7. Hybridization of the L, M, and S fragments to labeled vector (Figure 7) identified which fragments contain the 5' and 3' flanking pieces of the vector. The pBR322 probe hybridized to the M and S pieces in lanes 1 and 3 of the autoradiograph in panel B. Total pHpaCa7 cut with MspI, blotted and probed with pBR322, gave identical M and S band signals (data not shown). These data indicated that the vector 5' 438 bp and 3' 136 bp ends are attached to the M and S fragment (but not specifically to which one) and not attached to the L fragment. No hybridization of pBR322 to the L piece indicated that the L fragment was an internal insert piece and consists of only C. albicans DNA. GEL program (307) estimates obtained from various measurements of the three largest MspI fragments of pHpaCa7 are listed in Table 3. The M and S fragment sizes listed in Table 3 are from sizing the combination pBR322-C. albicans fragments.

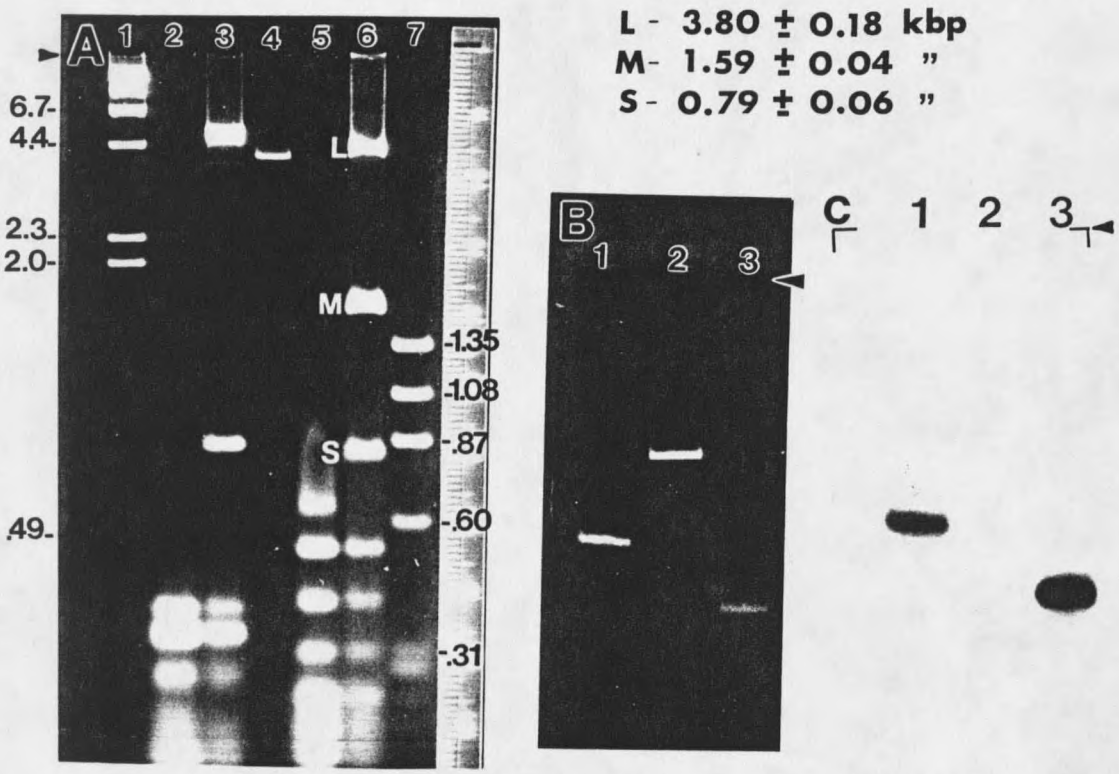


Figure 7. Comparative MspI digests of pHpaCa7 and pBR322. Panel A: MspI cut samples were electrophoresed in 2% TAE agarose. Lane 1 - Lambda HindIII marker; Lane 2 - pBR322 x HhaI; Lane 3 - pHpaCa7 x HhaI; Lane 5 - pBR322 x MspI; Lane 6 - pHpaCa7 x MspI; Lane 7 - ϕX174 x HaeIII marker; and Lane 4 - DEAE purified L fragment from pHpaCa7 x MspI. Lane 6 displayed 3 bands larger than lane 5 (denoted L, M, and S). Panel B: EtBr stained 1% TAE minigel of Geneclean purified L, M, and S fragments. Lane 1 - M; Lane 2 - L; and Lane 3 - S fragment. Panel C: Minigel blot was probed with labeled pBR322 and shows hybridization signals for only M and S lanes.

Revision of the restriction map of pHpaCa7, Figure 6 (99), was necessary to reflect the presence of the M and S fragments in the clone. Restriction data for ClaI, PstI, and HaeIII (but not EcoRI) digestion of the L fragment was re-evaluated without incorporating the pBR322 flank information. PstI digestion of whole pHpaCa7 gave two pieces, 1.8 kbp and 8.0 kbp. The sizes indicated pHpaCa7 insert orientation such that the S piece was 5' of L, and that the PstI site within L was closest to

the PstI site in pBR322 (at bp 3613, data not shown). HhaI digested pHpaCa7 (eg., lane 3, Figure 7) was compared with a MspI + HhaI double cut. The L fragment was not digested by HhaI but the M fragment yielded piece lengths compatible with a 3' end position for the M fragment.

Table 3. pHpaCa7 Insert Fragment Length Estimates

Fragment	Gel Number[1]	SD[2]	SC[3]	Length[4]
Large (L)	P132bk3	0.03	0.12	3.81
	P134bk3	0.03	0.13	3.80
	P67bk7	0.02	0.06	3.57
	P70bk7	0.02	0.14	4.01
	Sample Mean = 3.80 ± 0.18			
Medium (M)	P66bk7	0.06	0.16	1.60
	P72bk7	0.04	0.11	1.55
	P115bk4-2%	0.01	0.04	1.62
	Sample Mean = 1.59 ± 0.04			
Small (S)	P115bk4-2%	0.01	0.04	0.83
	P66bk7	0.04	0.16	0.73
	P67bk7	0.07	0.26	0.74
	Sample Mean = 0.77 ± 0.06			

[1]Gel Number indicates the page and research book for reference purposes.
[2]SD = Standard deviation of fragment lengths for each gel.
[3]SC = Standard deviation of the C[I] (the least squares estimate) indicates how well the data for standard gel markers fit the reciprocal mobility algorithm used in the GEL program.
Sample means were determined with a statistical calculator using s_{n-1} for SD.
[4]Length estimates expressed in kilobase pairs (kbp).

Figure 8 indicates a modified restriction map of pHpaCa7 with the 5' to 3' arrangement of S, L, and M, respectively. The possible presence of additional MspI fragments smaller than 150 bp (see Figure 7) in the pHpaCa7 insert region was not pursued by restriction enzyme or labeling analysis. Therefore, the map remains tentative. Using the average size values for the fragments (Table 3) and the known size of vector pieces attached, the S fragment would have 483 bp of pBR322 and M has 136 bp.

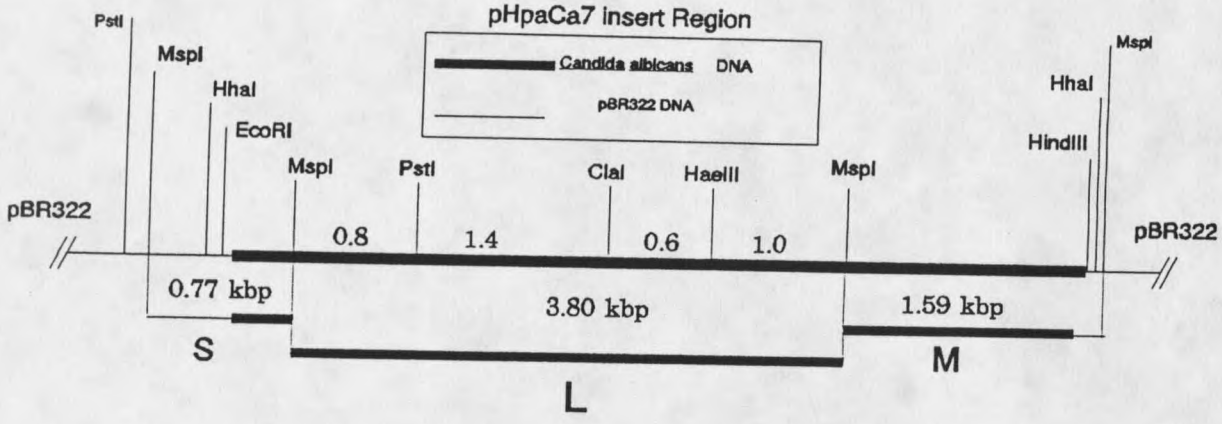


Figure 8. Modified restriction map of the pHpaCa7 insert. Sites are diagrammed with the 5' (EcoRI-side) and 3' (HindIII-side) flanking regions of pBR322. The presence of additional small MspI pieces remains possible, as mentioned in the text. Approximately 0.3 kbp of S and 1.45 kbp of M are C. albicans bases.

Genomic RFLP Analysis of L Fragment

Labeled L fragment was used to probe blots of MspI digested genomic DNA of 50 isolates of C. albicans and three of C. stellatoidea. Four patterns of bands, designated ♦I through ♦IV, were observed as shown by 10 example isolates in Figure 9. The 50 DNA samples included isolates from serogroups A (18) and B (20) (313) with no apparent correlation of serotype and hybridization pattern (Pattern ♦I - 6 A, 15 B, 3 unknown; ♦II - 2 A, 2 B, 1 unknown; ♦III - 6 A, 4 B, 1 unknown; and ♦IV - 4 A, 3 unknown). Serotype information was not available for the rest of the C. albicans strains. A doublet band pattern, ♦I, was predominant (25 of the 50 isolates) and three singlet band patterns were less frequent: ♦II, large size singlet (6/50); ♦III, medium size singlet (12/50); and ♦IV, small size singlet (7/50). C. albicans 9938 (DNA source for pHpaCa7 insert), exhibited pattern ♦III (Figure 9, lane

8). The C. stellatoidea DNA patterns (lanes 9-11: ♦I, ♦II, and ♦III) were the same as C. albicans signals. Table 4 lists the GEL program estimates of band lengths. Two singlet sizes, ♦II and ♦III, correlated in size with the two bands of pattern ♦I.

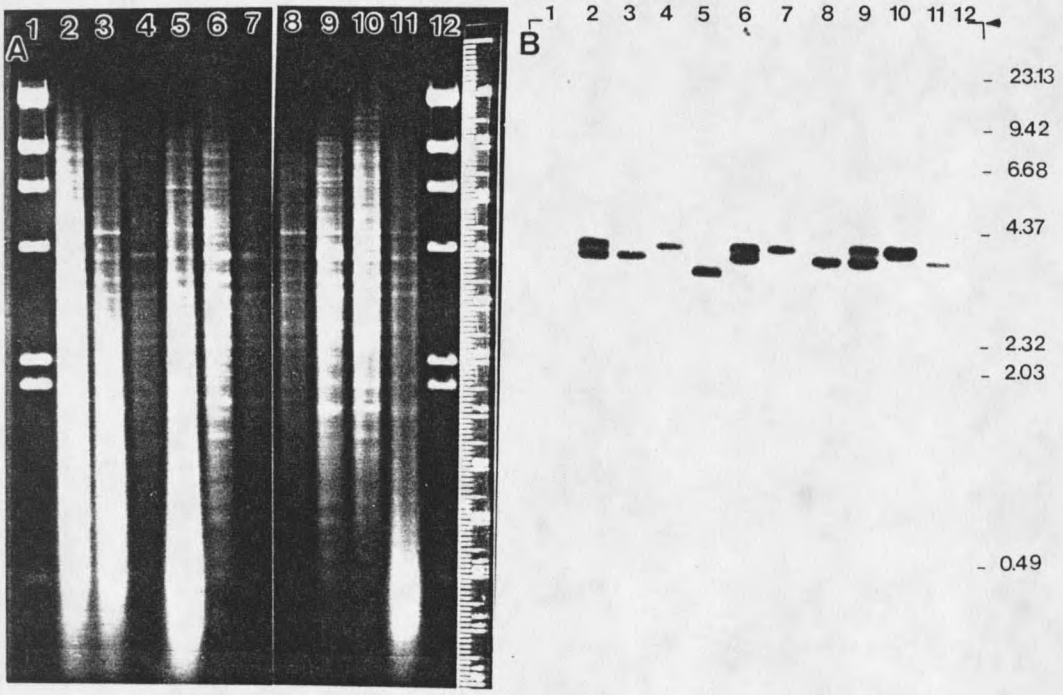


Figure 9. RFLP patterns of L hybridization. Panel A: EtBr stained 1% TAE gel. Lanes 1, 12 - Lambda x HindIII marker; Lanes 2-8 are MspI-digested C. albicans Ca1, B311 (ATCC 32354), 298, Ca6, 105, CaJ, and 9938 DNA, respectively. Lanes 9, 10 and 11 are C. stellatoidea isolates ATCC 4252 (Type I), ATCC 4365 (Type II), and ATCC 36232, respectively. (Type I - II C. stellatoidea refer to their karyotype, biochemical profile, and virulence characteristics (10,277)). Panel B: Southern blot probed with labeled L indicates hybridization bands for the doublet pattern ♦I in lanes 2, 6, and 9; pattern ♦II in lanes 4, 7, and 10; pattern ♦III in lanes 3, 8, and 11; and pattern ♦IV in lane 5.

Table 4. L Fragment RFLP Size Estimates

Gel Number[1]	SD[2]	SC[3]	Band Pattern Lengths[4] ◊I		◊II	◊III	◊IV
P140bk4	0.01	0.01	4.19	3.90	4.19	3.86	3.54
			4.13	3.90	4.13	3.84	3.59
			4.13	3.84		3.79	3.56
P139bk4	0.02	0.05	4.11	3.86	4.24	3.89	3.48
			4.18	3.92			
P150bk4	0.01	0.03	4.26	3.99	4.23	3.96	3.60
			4.16	3.90	4.16		3.74
Pattern Sample Means			◊I : 4.16 ± 0.05 and 3.90 ± 0.05				
			◊II : 4.19 ± 0.05				
			◊III : 3.87 ± 0.06				
			◊IV : 3.58 ± 0.08				

[1]Gel Number indicates the page and research book for reference purposes.
[2]SD = Standard deviation of fragment lengths for each gel.
[3]SC = Standard deviation of the C[I] (the least squares estimate) indicates fit for the reciprocal mobility model (GEL program).
[4]Length estimates are expressed in kbp; multiple values obtained from measurements of separate lanes.
Sample means were determined with a statistical calculator (s_{n-1} for SD).
Pattern sample sizes indicate the number of measurements taken per gel.

Extended Hybridization Survey of the L Fragment

Primary screening of *Candida* species DNA, digested with a four-base cutter, indicated that only *C. albicans* and *C. stellatoidea* genomic DNA reacted with the L fragment probe (99). To insure that small molecular weight *Msp*I fragments were not missed in the analysis, six-base cutters *Xho*I (C↓TCGAG) and *Eco*RI were chosen to execute fewer genomic cuts and generate larger average fragments than *Msp*I. Any potential target sequences from other samples would be less likely electrophoresed off the gel and not blotted. *Xho*I-digested *Candida*, *S. cerevisiae*, and *N. crassa* DNAs were blotted and probed with L (Figure 10). The probe hybridized only to *C. albicans* DNA (lane 2, panel B).

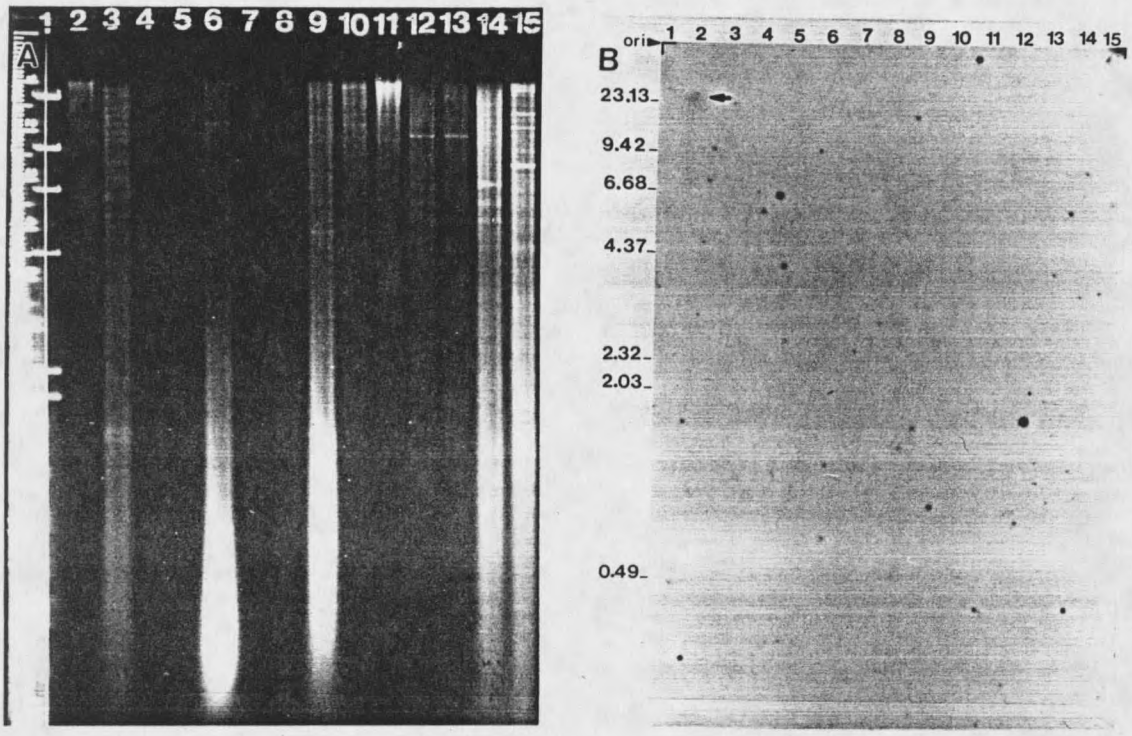


Figure 10. Gel and Southern blot of digested Candida species probed with the L fragment. Panel A: XhoI-digested DNAs were separated in 1% TAE agarose gels and blotted. Lane 1 - Lambda HindIII marker, Lane 2 - C. albicans 9938, Lanes 3 -C. lusitaniae, Lanes 4 & 5 are blank, Lane 6 - C. krusei, Lane 7 - C. tropicalis 1, Lane 8 - C. pseudotropicalis 2 (C. kefyr), Lane 9 - C. guilliermondii, Lanes 10 and 11 - C. parapsilosis 9 and 11, Lanes 12 and 13 - Torulopsis glabrata 1 and 2, Lane 14 - S. cerevisiae, Lane 15 - N. crassa AR33. Panel B: Autoradiograph exposed for 72 h at -70^0 C. The only signal was C. albicans 9938 DNA in lane 2 (arrow).

EcoRI-digested DNA from other medically important fungi (Table 1) were blotted and probed with the labeled L fragment (Figure 11). Of the DNA samples received as gifts, the A. flavis DNA was not susceptible to enzymatic cleavage even after SEVAG extraction and EtOH precipitation. The sample was subsequently sheared mechanically to provide for electrophoretic separation of fragments as seen in panel A (lane 7,

Figure 11). Again, the only hybridization was to C. albicans 9938 DNA (lane 10) which appears to be a partial EcoRI digest. The DNA samples in lanes 5 and 6 were loaded with 0.8 µg and 1 µg each; other L-probed blots with twice that amount remained negative (data not shown).

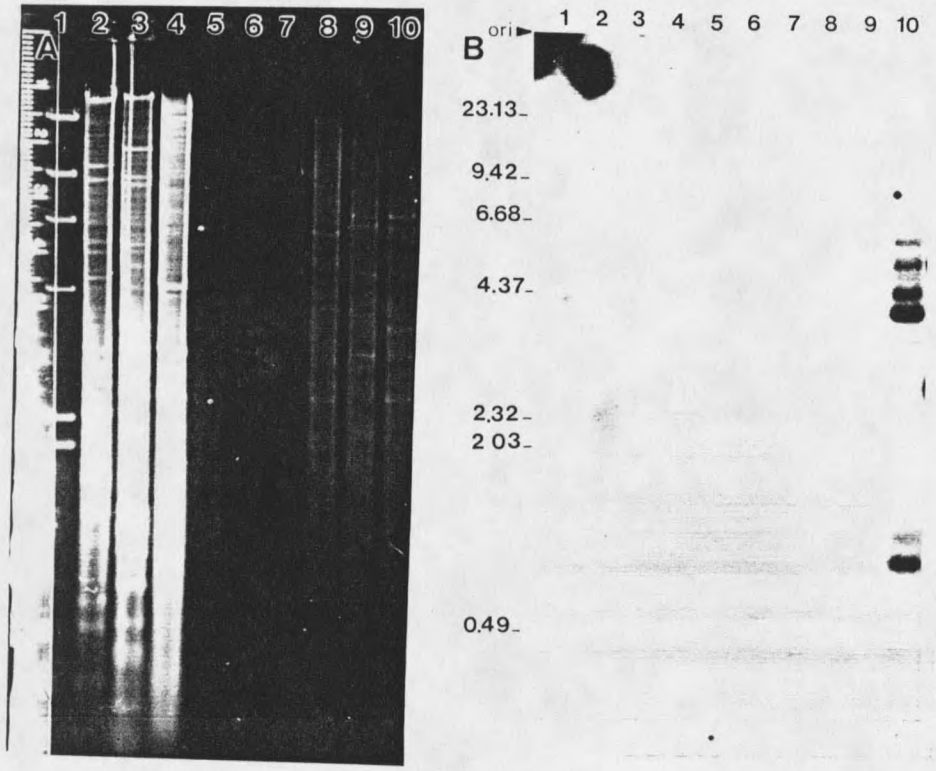


Figure 11. Gel and Southern blot of other medically important fungal DNA probed with L fragment. Panel A: Genomic DNA from various fungi were digested with EcoRI (except Aspergillus flavis which was sheared) and run on 1% TAE agarose and blotted to Genescreen Plus. Lane 1 - Lambda HindIII marker, Lane 2 -Histoplasma capsulatum G222B ATCC 26034, Lane 3-Blastomyces dermatitidis CR, Lane 4-Sepedonium chrysospermum ATCC 16084, Lane 5 - Chrysosporium keratinophilum ATCC 14803, Lane 6-Cryptococcus neoformans H99, Lane 7 - Aspergillus flavis H125, Lane 8 - Mucor racemosus 1216B, Lane 9 -Coccidioides immitis, Lane 10 - C. albicans 9938. Panel B: Hybridization signals for L are present only for C. albicans 9938 in lane 10. Also, Trichomonas vaginalis, was negative for L hybridization (data not shown). The spots in the upper left side of the blot are artifact and not seen in duplicate blots of H. capsulatum, B. dermatitidis, and S. chrysospermum DNAs probed with labeled L fragment (data not shown).

Hybridization Patterns Due to M and S Fragments

M and S Mixture Probe. During the Southern blot survey of DNA samples from various Candida species and other fungi (as in Figures 10 and 11), probing done with labeled, whole pHpaCa7 revealed hybridization to C. lusitaniae, C. krusei, C. parapsilosis, C. tropicalis, C. pseudotropicalis, C. guilliermondii, T. glabrata, S. cerevisiae, N. crassa, Histoplasma, Blastomyces, Sepedonium, Cryptococcus, Coccidioides, Mucor, and Aspergillus DNA (data not shown, T. vaginalis lanes were inconclusive). Human and mouse DNA samples were not included for probing with whole pHpaCa7.

To investigate the difference between L fragment and whole pHpaCa7 hybridization, Southern blots were probed with a mixture of Geneclean purified, labeled M and S fragments. Figures 12 and 13 show the M plus S hybridization of Candida species and other fungi. In figure 12, the panel B autogradiograph shows hybridization of the M + S probe mixture to high molecular weight XhoI fragments for C. albicans (lane 2), C. parapsilosis (lanes 8 and 9), and T. glabrata (lanes 10 and 11). The actual size of the hybridization positive XhoI fragments are uncertain because the EtBr staining patterns suggest incomplete cutting for lanes 2, 8, and 9. C. lusitaniae had a single band (lane 3), and C. tropicalis, C. pseudotropicalis, C. guilliermondii, S. cerevisiae, and N. crassa (lanes 5 - 7, 12, and 13, respectively) had two or more bands which hybridized to the mixture probe.

The M + S probe mixture exhibited hybridization to two Lambda HindIII marker fragments (Figure 13, lane 1), multiple bands in H.

capsulatum, B. dermatitidis, and S. chrysospermum (Figure 13, lanes 2 - 4), and fewer bands for C. keratinophilum, C. neoformans, M. racemosus, and C. immitis (lanes 5, 6, 8, and 9, respectively). The A. flavis signal in lane 7 appeared disperse due to sheared DNA. Loading of DNA samples was uneven across the lanes in Figure 13, therefore, additional bands in lanes 5 - 10 might be detected if more DNA were added. The weakest bands are marked with arrows to indicate the position seen on the original autoradiograph.

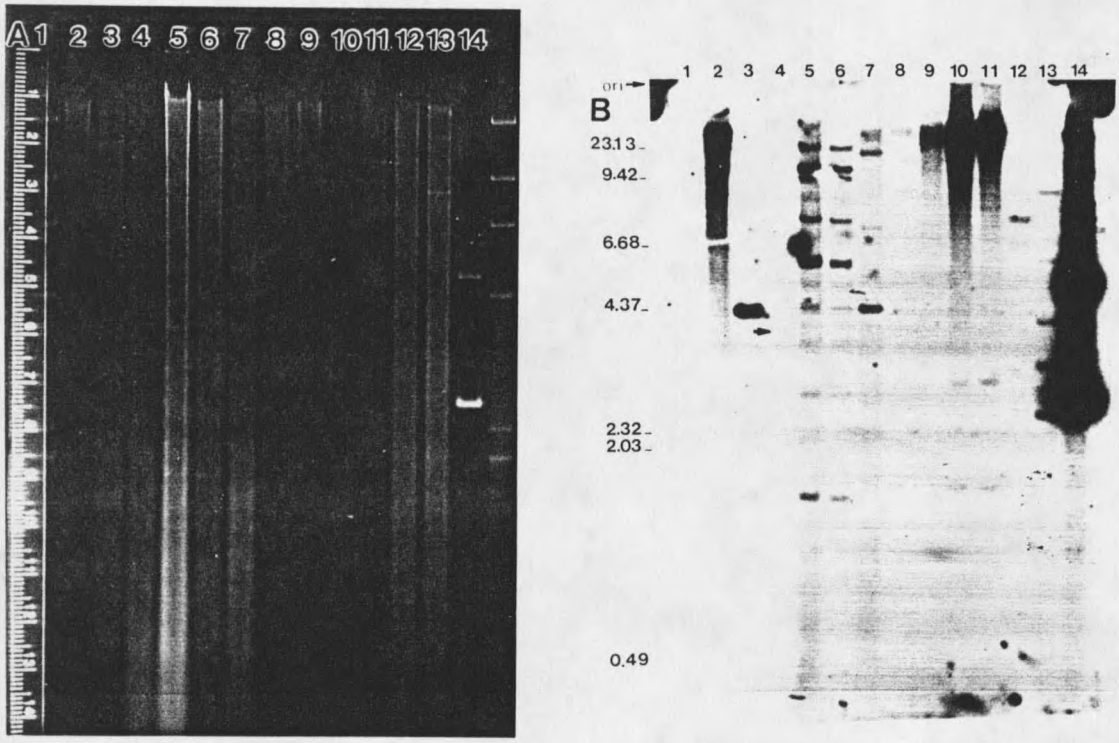


Figure 12. Hybridization patterns of fungal DNA probed with labeled M plus S fragments. Genomic DNAs were XhoI-digested, separated on 1% TAE gels, blotted, and probed with a mixture of labeled M and S fragments. Panel A: Lane 1 - Lambda HindIII marker; Lane 2 - C. albicans 9938; Lane 3 - C. lusitaniae; Lane 4 - C. krusei; Lane 5 - C. tropicalis; Lane 6 - C. pseudotropicalis, Lane 7 - C. guilliermondii; Lanes 8 and 9 - C. parapsilosis; Lanes 10 and 11 - T. glabrata; Lane 12 - S. cerevisiae; Lane 13 - N. crassa; and Lane 14 - uncut pBR322. Panel B: Autoradiograph of blot shows M and S probe hybridization to all DNA lanes. An arrow marks the weak band position in lane 4.

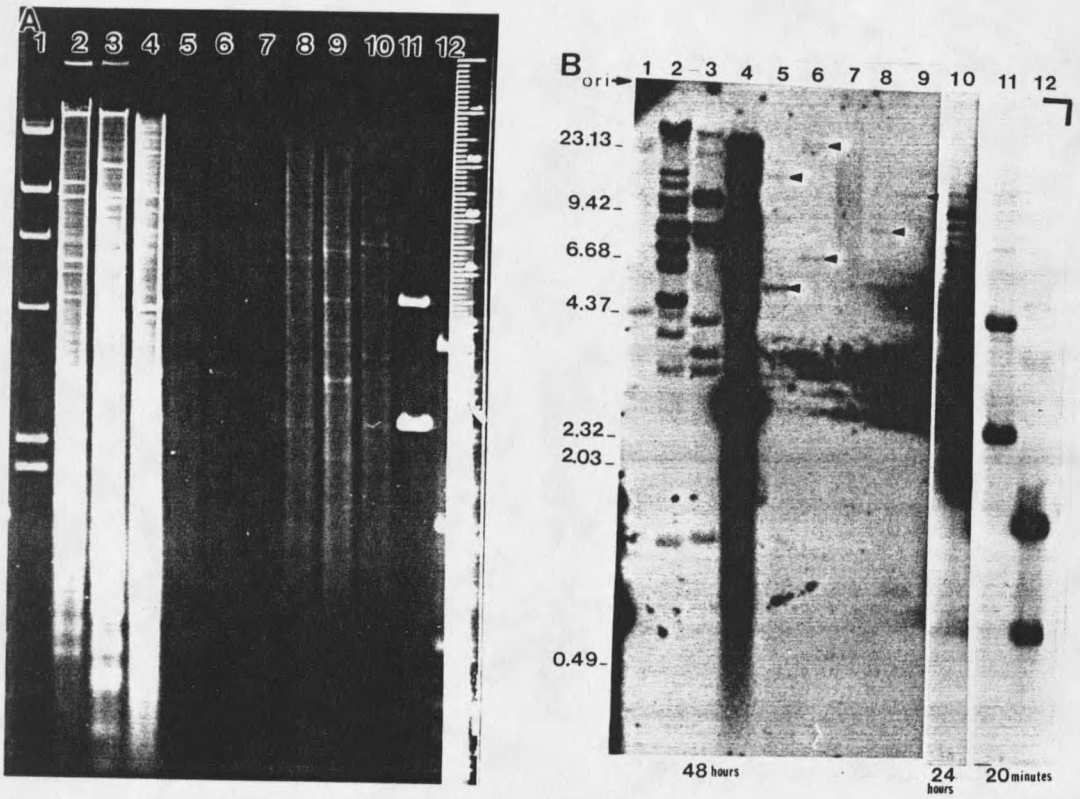


Figure 13. DNA hybridization patterns of additional medically important fungi probed with M plus S fragments. Fungal DNAs were EcoRI digested and separated in 1% TAE agarose. Panel A: Lane 1 - Lambda HindIII marker, Lane 2 - H. capsulatum, Lane 3 - B. dermatitidis, Lane 4 - S. chrysospermum, Lane 5 - C. keratinophilum, Lane 6 - C. neoformans, Lane 7 - A. flavis (sheared), Lane 8 - M. racemosus, Lane 9 - C. immitis, Lane 10 - C. albicans 9938, Lane 11 -pBR322, and Lane 12 - pHpaCa7 digested with MspI. Panel B: The Southern blot probed with M and S mixture was exposed for different times. Autoradiograph image of lanes 1 - 9 was exposed 48 h, lane 10 - 24 h, and lanes 12 and 13 were 20 min. M and S hybridized to all sample lanes, the weak signals in lanes 5, 6, 8, and 9 are marked by arrows. The signal in lane 7 appears as a smear because of shearing the A. flavis DNA. Two of the Lambda marker bands in lane 1 hybridized with the mixture probe.

Separate Probing with M and S Fragments. Results of hybridization by the S fragment or M fragment alone to the Candida species, Torulopsis, Saccharomyces, Neurospora, human, and mouse DNA are included

in Figure 14. C. krusei had one signal band (lane 3), the sheared human and mouse DNAs were both positive (lanes 10 and 11), and the other DNAs had at least 3 bands of hybridization. The M probe hybridized to C. krusei, C. guilliermondii, C. parapsilosis, S. cerevisiae, and N. crassa DNAs. The autoradiograph M signal image in lane 12 did not photograph well and is marked by an arrow.

Hybridization of the other medically important fungi (H. capsulatum, B. dermatitidis, S. chrysospermum, C. keratinophilum, C. neoformans, A. flavis, M. racemosus, and C. immitis) was also done using the separate probes, but insufficient DNA remaining in some donated samples prevented completing the separate M or S investigation. The S probe cross hybridized with H. capsulatum, B. dermatitidis, and S. chrysospermum, but the M probe did not (data not shown). No M or S probe signals were observed for Chrysosporium, Cryptococcus, Aspergillus, Mucor, or Coccidioides lanes that contained 0.8 - 1.5 µg DNA each (data not shown), even though all were positive with the combined M plus S probe when more DNA was blotted (Figure 13, lanes 5 - 9 with ≥ 2µg per lane).

Because the M and S fragments contain sequences from pBR322, duplicate Southern blots of the various Candida species, other fungi, human and mouse DNA were probed labeled pBR322. pBR322 DNA did not cross-hybridize with C. albicans or other Candida DNAs, but did hybridize to two lambda marker bands (23.13 and 4.37 kbp) and a band in Chrysosporium at 3.62 ± 0.03 kbp (GEL program estimate, data not shown) which was observed for the M + S probe (Figure 13, lanes 1 and 5).

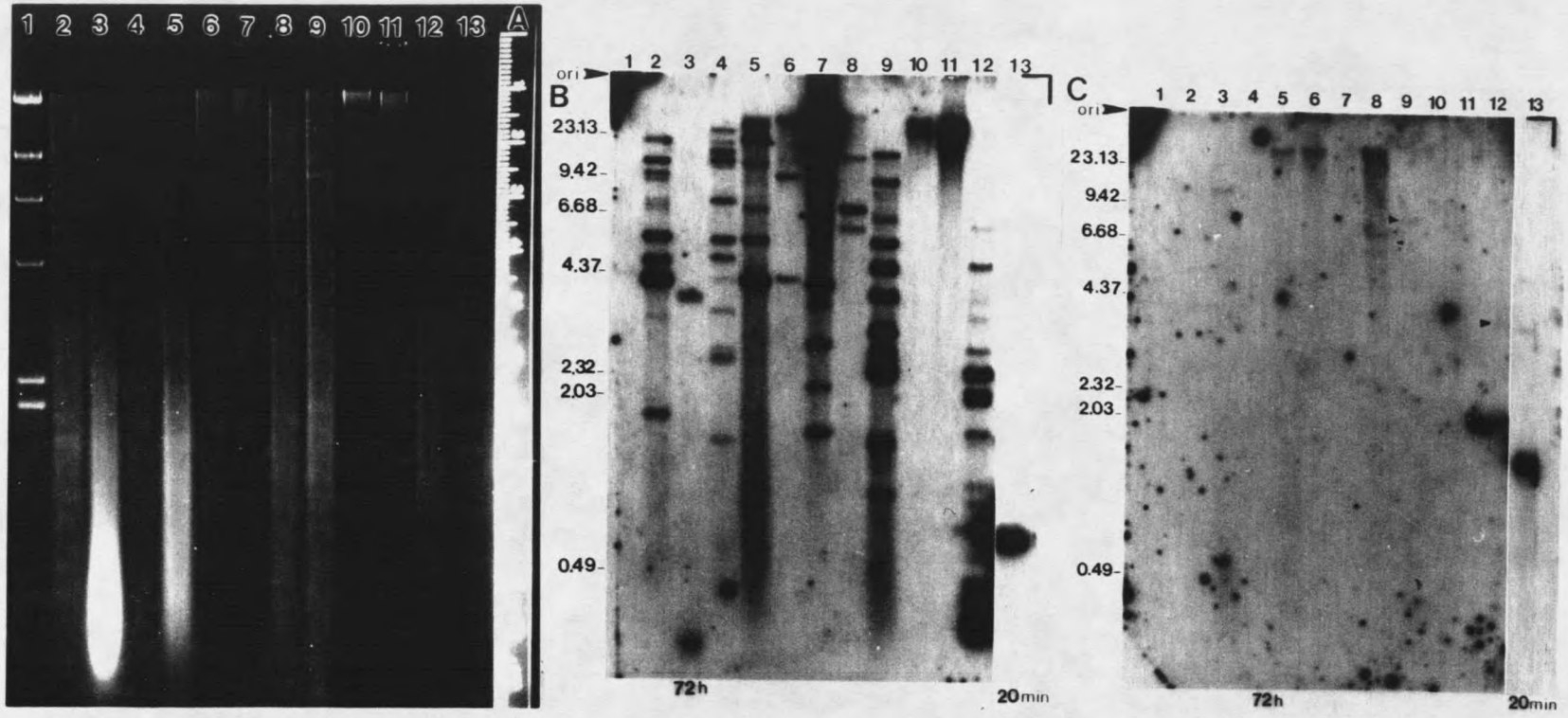


Figure 14. Gel and Southern blot of various species surveyed with M or S probe. XhoI digested DNAs were set up in duplicate on 1% TAE agarose, electrophoresed, and blotted to Genescreen Plus. Panel A: Photograph of right half (left half had identical loading). Gel Lanes 1 - 12 for each half: Lane 1 - Lambda HindIII marker, Lane 2 - C. lusitaniae, Lane 3 - C. krusei, Lane 4 - C. pseudotropicalis, Lane 5 - C. guilliermondii, Lane 6 -C. parapsilosis, Lane 7 - T. glabrata, Lane 8 - S. cerevisiae, Lane 9 - N. crassa, Lane 10 - human (sheared), Lane 11 - mouse (sheared), Lane 12 - C. albicans 9938, and Lane 13 - MspI-digested pHpaCa7. Panel B: Autoradiograph of blot probed with S fragment showing all lanes positive. Panel C: Autoradiograph of M probed blot indicating hybridization to lanes 3, 5, 6, 8, and 9 (arrow). The 9938 hybridization signal was weak and is also marked with an arrow in lane 12. The pHpaCa7 lane of panel B and C were cut off and exposed for a shorter time than the rest of the blot.

RFLP Analysis with the S fragment

XhoI-digest C. albicans 9938 probed with the S fragment (Figure 14, panel B, lane 12) exhibited more than 7 signal bands. To determine if the S fragment would exhibit RFLPs among various isolates of C. albicans, Southern blots of MspI digested DNAs were prepared. DNA samples were electrophoresed in groups according to L probe patterns ◊I through ◊IV (Figure 15): pattern ◊I isolates are in lanes 2-4; ◊II in lanes 5-8; ◊III in lanes 9-11; and ◊IV in lanes 12-14. DNA in lanes 15-20 were not grouped. Panel B of Figure 15 shows that the S probe hybridized to 5 bands in lane 2 (arrowheads) and 10 bands in lane 13 (arrowheads) for different MspI-digested DNA samples.

In each of the L pattern groups, different S probe profiles were observed: ◊I - lanes 2, 3, 4, and 18 all differ; ◊II - lanes 6 and 7 are similar but different than lanes 5, 8, and 19; ◊III - all lanes (9-11, 15) differ; and ◊IV - all lanes differ (12 - 14, 20). Also, isolates from different L group patterns displayed the same S profile. For example, the following lanes appeared similar in Figure 15: lanes 2 and 10 (◊I,◊III), lanes 3 and 5 (◊I,◊II), and lane 4 and 6,7 (◊I,◊II).

Panel A (Figure 15) exhibits a number of MspI fragments larger than 2 kbp as mentioned for Figure 1. The group of three intense bands similar for all C. albicans isolates is marked with arrowheads between lanes 1 and 2 (Figure 15, panel A). The autoradiograph (panel B) indicates S signals at approximately 4.2 kbp (top arrowhead, lane 2) for some isolates, but are absent in lanes 9 - 12, 14, 15, 17, and 20. Therefore, the S fragment may contribute, but is not solely responsible for, the observed EtBr intensity of the 4.2 kbp band.

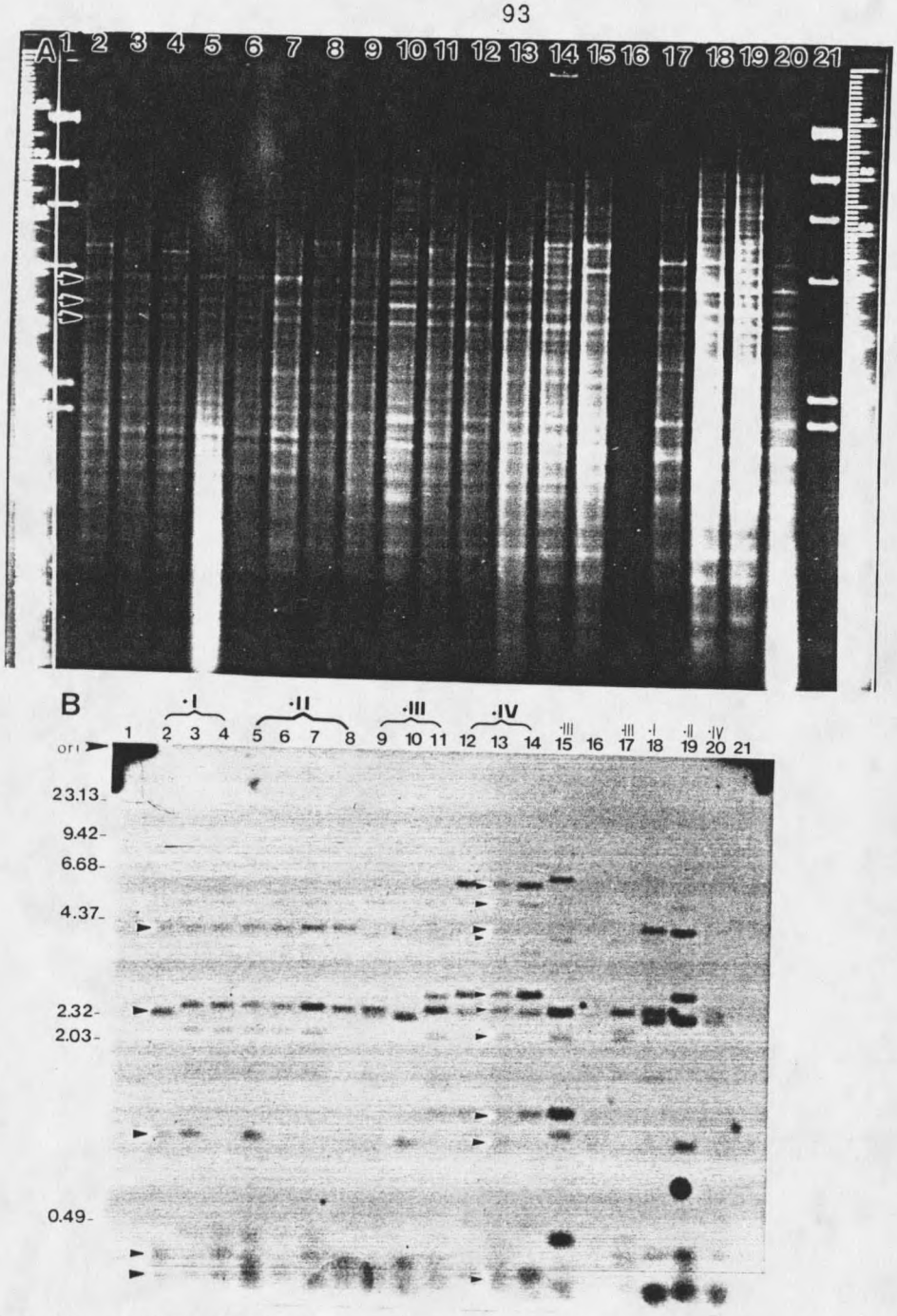


Figure 15. RFLP of the S fragment to MspI-digested C. albicans DNA samples. Panel A: EtBr stained 1% TAE gel of MspI cut DNAs. Lanes 1 and 21 are Lambda HindIII marker fragments. L Pattern ♦I: lane 2 - Ca 220, lane 3 - Ca253, lane 4 - Ca295; L Pattern ♦II: lane 5 - Ca23, lane 6 - Ca298, lane 7 - CaJ, lane 8 - Ca325; L Pattern ♦III: lane 9 - Ca207, lane 10 - Ca220, lane 11 - 228; and L Pattern ♦IV: lane 12 - Ca 202, lane 13 - Ca213, lane 14 - Ca313. Other lanes: lane 15 - CaOs (L pattern ♦III), lane 16 - Ca1 (DNA sample lost, well broke), lane 17 - Ca 9938 (L pattern ♦III) and lanes 18 - 20 are C. stellatoidea ATCC 4252 (♦I), ATCC 4365 (♦II), and ATCC 36232 (♦IV).

Hybridization of L Fragment to Total and mtDNA

To determine if the C. albicans and C. stellatoidea specific DNA fragment L was cloned from nuclear or mtDNA, blots of C. albicans genomic and mtDNA 1 were probed with labeled L fragment (Figure 16). Nuclear origin of L was indicated by the absence of a mtDNA signal and a positive signal for total DNA. Additional bands are visible in lower molecular weight MspI sizes (lane 1 < 3.6 kbp) and appear similar to S fragment signals in Figure 15. This L fragment probe was prepared from a partial MspI digest of pHpaCa7, prior to knowing that the M and S fragments were present in pHpaCa7. Hybridization of the M or S fragment to purified mtDNA were not done, but CHEF blots of Candida chromosomes indicates M and S fragments present in the nucleus (Fig.19, panels B,D).

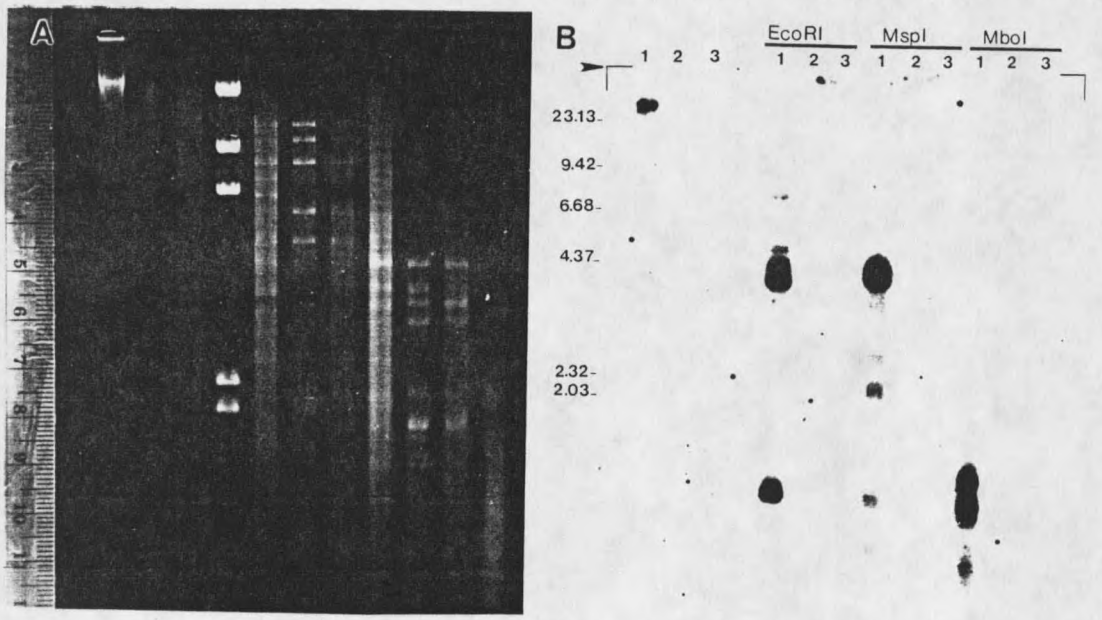


Figure 16. Gel and Southern hybridization of the L fragment to total and mtDNA. Panel A: Ca1 total and mtDNA samples were digested with EcoRI, MspI, or MboI and separated in 1% TAE agarose and blotted. The first three lanes contain undigested preparations, followed by lambda HindIII marker DNA, and lanes for the enzyme digested samples. Lane 1 - total DNA, Lane 2 - mtDNA, and Lane 3 - "heavy" mtDNA as described in Appendix C. Panel B: Autoradiograph of blot showing hybridization only in the lanes containing total DNA (lane 1 of group).

L Hybridization During Morphogenesis

The isoschizomers, MspI and HpaII, are commonly used to detect 5-methylcytosine (5mC or ^{m}C) changes in eukaryotic DNA. MspI cleaves CCGG or C^{m}CGG, but does not cut ^{m}CCGG sequences ~{2478}~. HpaII digests CCGG or ^{m}CCGG but not C^{m}CGG. Because 5mC changes in 5' promoter regions are associated with some instances of gene regulation and developmental changes (314), and because the L fragment may A-T rich similar to yeast promoter regions (315,316), the genomic L size was analyzed by Southern blot for possible 5mC changes in yeast versus mycelial DNA.

C. albicans 9938 was germinated by the mLee medium temperature-shift induction method on three separate occasions. The first 9938 culture (January 1989) produced true germ tubes and was used for DNA extracts shown in Figure 17. The second and third cultures (April 1990 and May 1991) yielded primarily pseudohyphal cells (>80% by microscopic examination) and were used for RNA extraction (Figure 21). To check for methylation changes during morphogenesis, DNA from 9938 yeast and filamentous forms were digested with MspI or HpaII and probed with labeled L fragment (Figure 17). No L signal shift was observed (panel B) which suggests two characteristics of the genomic L fragment.

First, the original 9938 L fragment was from a HpaII digest of genomic DNA, therefore the cytosines of the terminal CCGG sites would either be unmethylated or only the first could be 5mC to allow HpaII digestion. Comparing lanes 1, 2, 4, and 5 in panel B of Figure 17, the L fragment was the same size regardless of MspI or HpaII digestion of genomic DNA indicating that both enzymes acted at the same site which would only occur when the CCGG sequences at the ends of the genomic L

fragment do not contain 5mC residues. Also, because the genomic size of the L fragment does not vary in the yeast versus mycelial lanes of Figure 17 (lanes 1, 2, 4, 5 versus 3 and 6) the terminal CCGG sites remain unmethylated during morphogenesis. When yeast and mycelial cell DNA from three other isolates (Ca1, CaOs, and Ca105) were similarly compared, L hybridization sizes remained constant (data not shown). M and S probing of yeast and mycelial DNA blots was not done. (See Appendix A for growth of C. albicans in demethylating agents.)

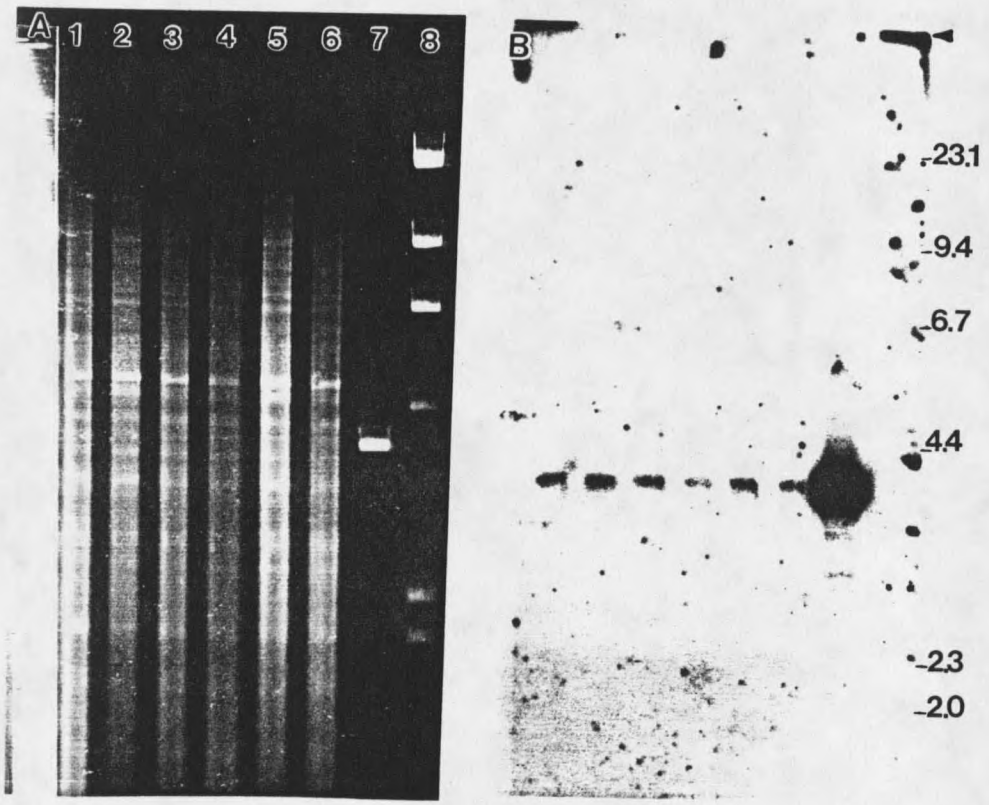


Figure 17. Gel and Southern blot of yeast and mycelial form DNA probed with L fragment. DNA samples digested with MspI (Lanes 1-3) or HpaII (Lanes 4-6) were electrophoresed on 1% TAE agarose and blotted. Panel A: Lanes 1, 4 - 9938 yeast form (GYEP); Lanes 2, 5 - 9938 yeast form (mLee); Lanes 3, 6 - 9938 mycelial form Lane 7 - DEAE purified L fragment; and Lambda HindIII marker in Lane 8. Panel B: Autoradiograph of L probed blot shows no size change for the genomic L fragment during germination.

Chromosomal Hybridization of L, M, and S Fragments

CHEF Electrophoresis. Electrophoretic parameters developed by Andreas Luder (personal communication regarding the same CHEF apparatus) for C. albicans were 48 h, 100 V, and 400 s pulses in 1% (0.5 x TBE) agarose. When the run time was lengthened to 60 h, better separation occurred for bands in the large chromosomal region. Replacement of circuitry controlling the alternating electrophoretic pulses (CGA/F "outside" switch box, M.J.Research, Watertown, MA) required a run time extension to 80 h for similar migration of the four smaller chromosomes but the resolution of the larger chromosomes was less than with the previous 60 h time.

The current estimate of chromosome number for most isolates of C. albicans is eight (differences described in the introduction), numbered from largest to smallest as R and 1 - 7, respectively (275). The CHEF results described here do not include strains published to have 8 chromosomes and were done prior to the publication of TAFE or CHEF run parameters for 8 chromosome separation (98,275). S. cerevisiae chromosomes were included in preliminary gels for size references for the smallest Candida chromosomes (data not shown) and Karla Dugan prepared CHEF gels containing both C. albicans and S. pombe DNA that provided size references for the larger Candida chromosomes (personal communication).

Assignment of putative chromosomal identification numbers (lane 6, Figure 18) was done by comparing the reported migration of Candida, S. pombe, and S. cerevisiae chromosomes under similar OFAGE and CHEF

conditions (10,11,317) to the chromosomes from all three fungi separated by this CHEF apparatus.

Labeled rDNA from N. crassa hybridized to the largest band of C. albicans 9938 on Southern blots from gels similar to those shown in Figures 18 and 19 (data not shown). Though other chromosome specific DNA probes were not used, the rDNA-containing chromosome (R) has shown variable migration position relative to only chromosomes 1 and 2 among C. albicans isolates (275). The smallest C. albicans chromosomes (4, 5, 6, and 7) are well separated (80 h, 100V, 400 s switch), chromosome 3 exhibits less separation, and the largest chromosomes (R, 1, and 2, assuming 8 total), remain unresolved under these CHEF conditions. Other investigators have shown that ramp switch modifications resolved more than six to seven chromosomal bands and produced separation of the chromosome containing rDNA and the other two large chromosomes from C. albicans, but the parameters were not tested for these isolates (98,275).

Other electrophoretic parameters were tested (strains 9938, CaA, and Ca1) for better separation of the large chromosomes: a.) 180V, 18 h, 120 sec switch followed by 100 V, 25 h, 300 sec switch time (Beth Hazen, personal communication) produced greater movement of the large bands, but not resolution of chromosomes R, 1, 2, and 3; b.) 100 V, 45 h, with a linear pulse ramp of 400-800 sec produced greater movement of chromosome 3 but not R, 1, and 2; and c.) 100 V, 54 h, 400 s switch followed by 80 V, 24 h, 1200 sec switch time exhibited a smear for chromosomes R, 1, and 2 (data not shown for the parameters a, b, or c).

CHEF gels containing DNA from yeast and mycelial forms of C. albicans isolates 9938, Ca1, and Ca105 did not indicate karyotypic changes associated with the yeast or mycelial form of growth (data not shown). Also, no individual karyotype changes were observed for strains 9938 and Ca1 (from refrigerated subcultures) tested 5 times at approximately 6 month intervals over a 3 year period (data not shown).

L, M, and S Chromosomal Hybridization. Chromosomal DNA from yeast forms of C. albicans strains Ca1, 9938, and CaA (57), were tested for L fragment hybridization. The actin gene (95) which hybridizes to chromosome 1 (275) was included for comparison as a single copy gene control. The actin probe in panel B of Figure 18 hybridized to the unresolved chromosome group (R, 1, and 2). The L probe hybridized to chromosomes 3 and 7 (panel C, Figure 18). Hybridization of CHEF Southern blots with labeled M and S probes are shown in Figure 19. The M probe hybridized to the unresolved large chromosomes (panel B). S fragment hybridization in panel D indicates signals from all EtBr stained bands on the gel: chromosomes 3 through 7, and the unresolved large chromosomal group R, 1, and 2.

These hybridization results suggest a minimal estimate of genomic copy number for the L, M, and S fragment. The L probe hybridized to 2 chromosomes indicating at least 2 copies per haploid genome. Because the M probe annealed to the unresolved large group of 3 chromosomes, the lowest copy number could be 1 - 3 copies per haploid genome. The S probe hybridized to five separated chromosomes and to the large group, suggesting at least six copies per haploid genome.

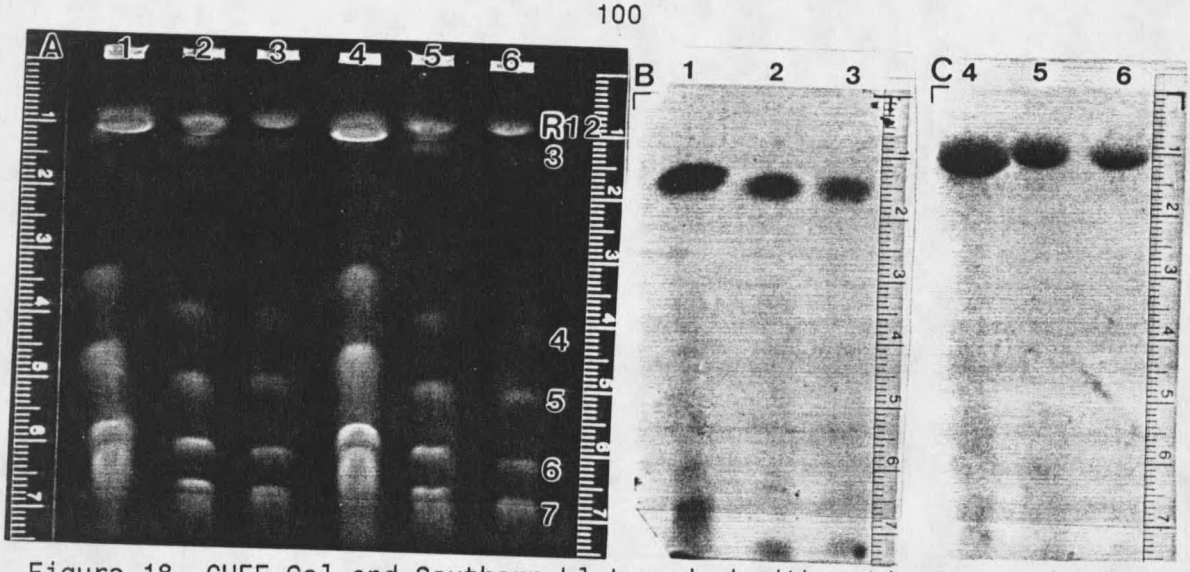


Figure 18. CHEF Gel and Southern blot probed with actin gene and L fragment probes. Panel A: C. albicans DNA samples were electrophoresed in 1% (0.5xTBE) agarose gel for 80 h, 400 s switch time, stained, divided in half,and blotted to Genescreen Plus membranes. Lanes 1,4 - 9938; Lanes 2,5 - CaA; Lanes 3, 6 - Ca1. Migration of 9938 chromosomes 4, 5, 6, and 7 (lanes 1 and 4) appear slower than the same chromosomes of Ca1 and CaA. Panel B and C: Autoradiograph of gel halves. The two blots were probed with L fragment probe (Panel B) or actin (Panel C).

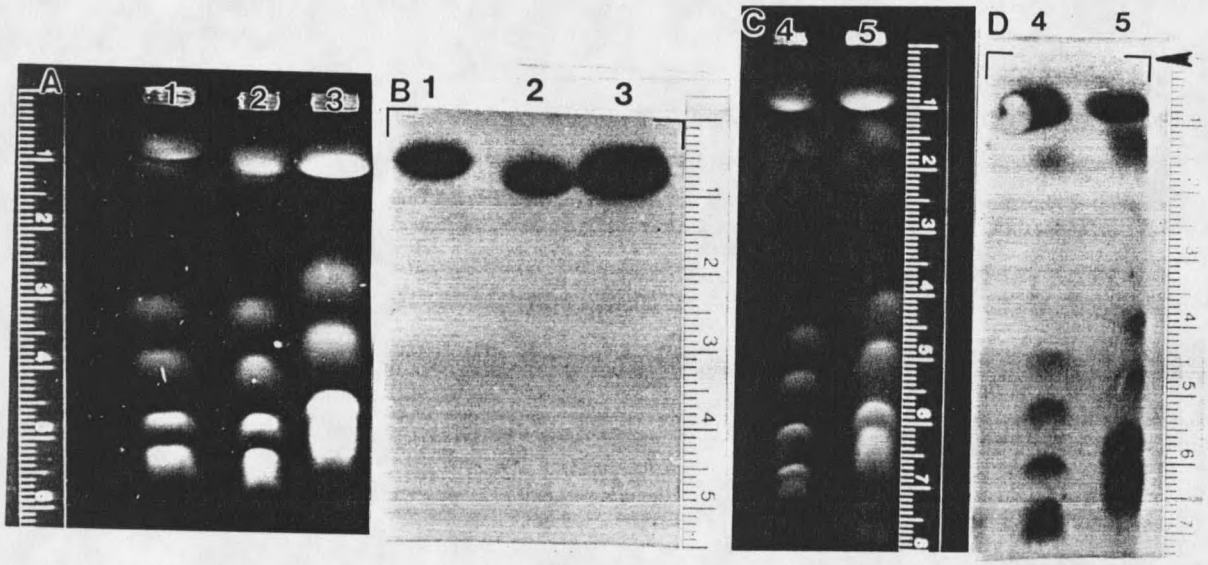
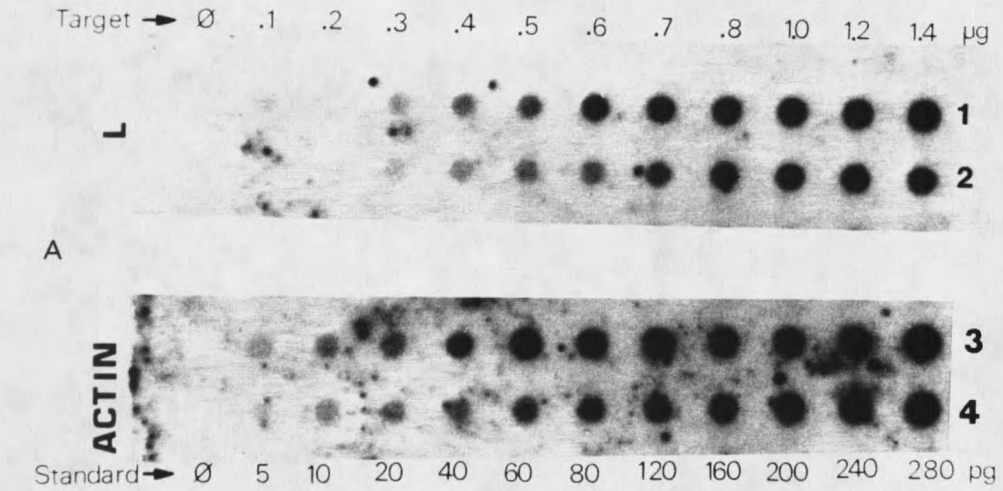


Figure 19. Chromosomal hybridization of M and S fragments. Samples were electrophoresed in 1% (0.5xTBE) agarose for 60h, 100V, 400s (panel A) or 80 h, 100 V, 400 s (panel C) switch time. Panels A and C: Lanes 1,4 - Ca1; Lanes 2,5 - CaA; Lane 3 - Ca 9938. Panel B: Autoradiograph of M probed lanes 1-3 shows hybridization to unresolved chromosomes R, 1, and 2. Panel D: Autoradiograph of S probed lanes 4 and 5. The S probe hybridized to individual chromosomes 3 - 7 and the group (R, 1, and 2).

Dot Blot Analysis of L Fragment Copy Number

The DNA content of C. albicans is approximately 40 fg/cell as reported values range from 35.5 - 41.9 fg/cell [reviewed in (239)]. For convenience, 40 fg/cell was used to calculate the weight of 1 x 10^7 diploid genomes as equal to 0.4 µg DNA (or 0.2 µg DNA for 1x10^7 haploid genomes). The following formula was used to convert the basepair lengths of the L fragment (3,790 bp) and actin gene (2,100 bp) into approximate weights per copy: grams = (bp length) x (308 daltons/nt) x (2 nt/bp) x (1.65 x 10^{-24} g /dalton). [The average dalton/nt value was obtained from an Oligo Technical Card (Genetic Designs, Inc.)]. The L fragment equals 3.85 x 10^{-18} g/copy and actin equals 2.13 x 10^{-18} g/copy. Multiplying by 1 x 10^7 gives a single copy correspondence estimated for 0.2 µg target DNA (per 1 x 10^7 haploid genomes) at 21.3 pg actin gene and 38.5 pg L fragment.

DNA dot blots were prepared with increasing picogram amounts of actin or L fragment DNA applied to separate Genescreen Plus membranes for standard curves of signal intensity. Each blot contained EcoRI-digested C. albicans 9938 DNA in microgram amounts for target DNA signals. The blots were probed with labeled actin or L fragment DNA (Figure 20). The autoradiograph blot images were scanned using a Microcomputer Imaging Device (MCID Version 2.6, Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada) calibrated to standard optical density (OD) units (scale provided by manufacturer). A linear regression plot of each standard curve appears in Figure 20 (Sigmaplot Version 4.1, Jandel Scientific). OD values for target DNA spots are listed in the box.



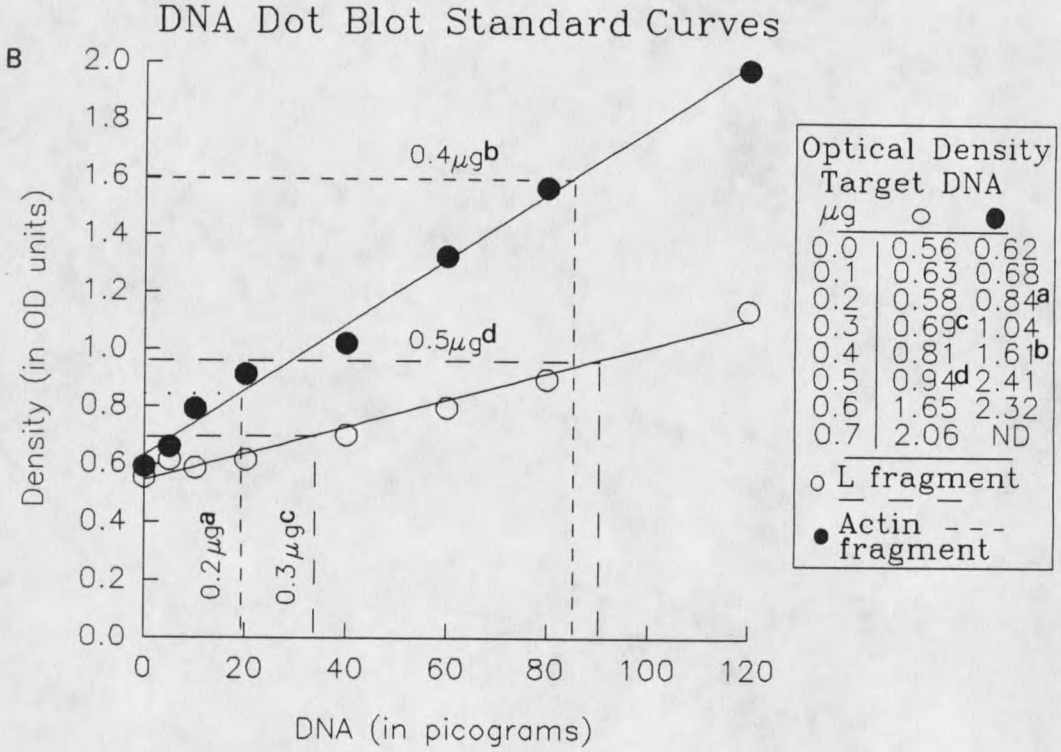


Figure 20. Dot blot hybridization of L fragment. Panel A: Autoradiographs of L and Actin dot blots. The L fragment (Row 2) and Actin DNA (Row 4) were applied in 0 - 280 picograms for standard curves, and EcoRI-digested 9938 target DNAs (0.1 - 1.4 micrograms) are in Rows 1 and 3. Panel B: Linear regression graph of MCID OD values plotted against standard DNA quantities of 0 - 120 picograms that had OD values on scale. Target DNA OD values are listed in the box. Two sample intercept lines, labeled a - d, were drawn to correlate the target DNA intensities with each standard curve. For actin: dotted line a - 0.2µg ≈ 20pg, dotted line b - 0.4µg ≈ 86pg; For L: dotted line c - 0.3µg ≈ 33pg, dotted line d - 0.5µg ≈ 90pg.

Two OD values from each set of target DNA in Figure R15 were drawn as graph intercept lines to indicate correspondence between target dot intensity and standard curve values. For the two actin dotted lines a and b, line a has a single copy (per haploid genome) correspondence of 0.2 µg target to 20 pg standard DNA which is very close to the estimated single copy 0.2 µg to 21.3 pg standard DNA. Line b has double copy values of 0.4 µg to 86 pg (or 0.2 µg to 42 pg). The estimated single copy value for L was 0.2 µg target to 38.5 pg standard. Line c has a 0.3 µg to 33 pg intercept, or 0.2 µg to 22 pg indicating a 0.57 L copy correspondence (22/38.5). Line d, at 0.5 µg to 90 pg (or 0.2 µg to 36 pg), has a 0.94 L copy per haploid genome correlation.

CHEF hybridization data and dot blot results indicate that the L fragment has a similar, low copy number like the actin gene. Two chromosomes (3 and 7) hybridized with the L probe, while the intercept lines c and d indicate single copy (or less) per haploid genome. These results suggest 1 - 2 copies of L per haploid genome.

C. albicans 1 (doublet L pattern ◊I), was also used as a target DNA for the dot blot assay, but a consistent signal strength difference from 9938 DNA (◊III singlet L pattern) was not obvious (data not shown). Dot blot copy number analysis was not done for M and S fragments.

Northern Hybridization Analysis of the L, M, and S Fragments

RNA samples from yeast, and pseudohyphal (C. albicans 9938) or mycelial forms (C. albicans 1) were examined to determine if the cloned candidal fragments contain sequences which are transcribed. In particular, the species specific hybridization of L and the widespread

hybridization of the S fragment provoke consideration of possible DNA function, but from opposite perspectives. The S fragment indicates broad species DNA conservation while the L fragment shows species maintenance of a unique DNA sequence.

Formaldehyde Agarose RNA Gels. Formaldehyde agarose gels were prepared and samples arranged to provide separate RNA lanes for EtBr staining or nitrocellulose blotting. Northern blots, from a low-concentration formaldehyde gel (methods section) were probed with labeled actin or L fragment DNA (Fig. 21, panel B and C, respectively). Lanes contained 10 µg total RNA (CsCl method), but because the 25S and 17S rRNA bands aren't visible, these samples may have been degraded. RNA samples prepared by acidic phenol extraction exhibit bands which may be rRNA bands (Figure 22, panel A, lane 2 arrows). Northern blots probed with labeled rDNA were not done to confirm rDNA band identities.

Probing with the L fragment resulted in weak signals for yeast, mycelial, and pseudohyphal total RNA samples (Figure 21, panel B, lanes 4 - 7 arrow). The positive hybridization results indicate that L fragment sequences are transcribed. Weak signal strength may result from low level expression of L sequences or, possibly, that not all of the coding region was included in the pHpaCa7 clone. DNAse treated 9938 RNA samples (methods section) were blotted and probed with the L and also exhibited weak signals at about 0.9 kb, in a formaldehyde gel that included an RNA ladder (GEL program estimate, data not shown).
Panel C of Figure 21 shows that the actin probe produced weak signals for yeast phase RNA (lanes 8 and 10), a strong signal for 9938 pseudohyphal RNA (lane 9), and a moderate signal for Ca1 mycelial RNA

(lane 11). Because total RNA amounts per lane were the same, the strong hybridization signal for 9938 pseudohyphal RNA suggested higher levels of actin expression than in C. albicans 1 mycelial cells (Figure 21, panel C, lane 9 versus 11). The full sequence of the single, C. albicans actin gene has been published (318) and actin mRNA levels have indicated a six-fold increase during the formation of true germ tubes from yeast forms (211). It is uncertain whether an actual difference exists between pseudohyphal actin expression and mycelial forms, or if this observation was peculiar to isolate 9938, but the signal differences were also observed in Northern dot blots (Figure 24).

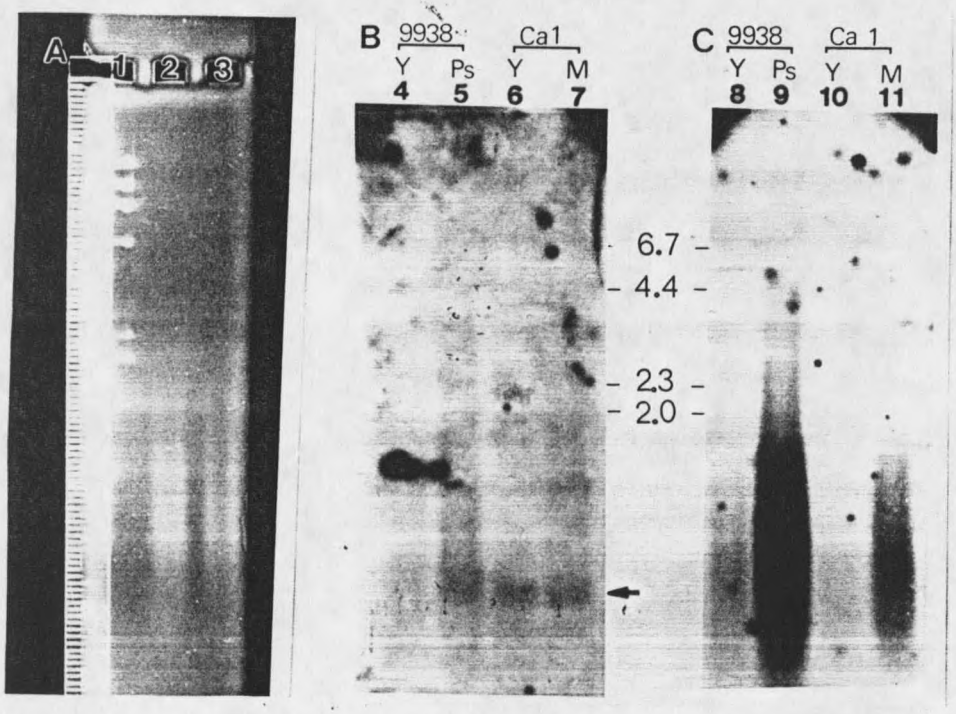


Figure 21. Gel and Northern hybridization of the L fragment and Actin to C. albicans RNA. RNA samples (CsCl method) electrophoresed in 1.2 % formaldehyde agarose gel were in separate lanes for staining (Panel A) or nitrocellulose blotting (Panel B and C). Panel A: EtBr stained gel. Lane 1 - Lambda HindIII DNA, Lane 2 - Ca 9938 yeast form, Lane 3 - Ca 1 yeast form. RNA samples in Panel B and C: lanes 4,8 - 9938 yeast form; lanes 5,9 - 9938 pseudohyphal form; lanes 6,10 - Ca 1 yeast form; lanes 7,11 - Ca 1 mycelial form. Panel B blot was probed with L DNA and panel C with actin gene DNA. Panel B arrow indicates L hybridization signal.

Hybridization of RNA samples to labeled M and S fragments are shown in panel B of Figure 22. The S fragment probe of total RNA yielded two large bands (8.48 and 6.14 kb, lanes 4, 5). Labeled M fragment probing produced a disperse signal smaller than the 1.35 kb RNA marker (Fig. 22, panel B, lane 6). DNAse treated total RNA samples also gave positive hybridization signals with both the M and S probes (Figure 24).

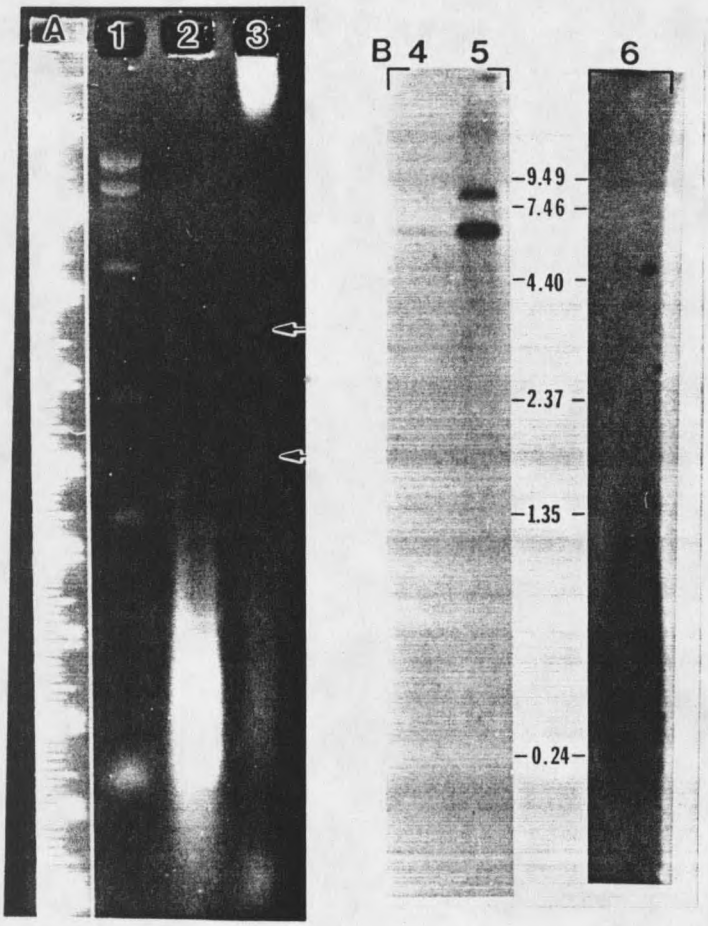
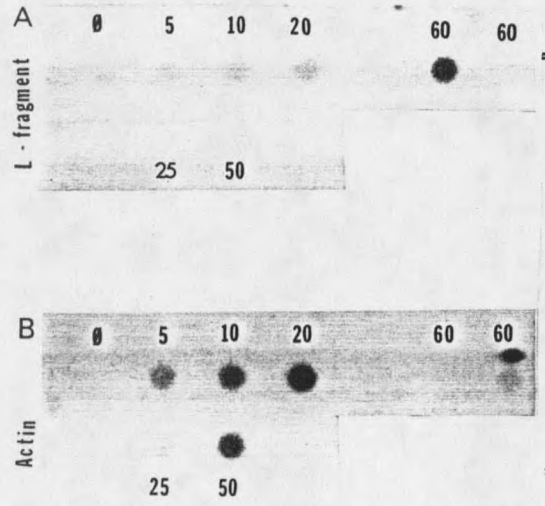


Figure 22. Gel and Northern hybridization of M and S fragment to C. albicans RNA. Yeast form total RNA samples (20 µg from CsCl method except for lane 3) were electrophoresed in 1.2% formaldehyde agarose gel and EtBr stained or blotted to nitrocellulose. Panel A: Gel stained with EtBr: Lane 1 - RNA size marker (BRL), Lane 2 - 9938, Lane 3 - 9938 (18µg, phenol method). Panel B: Autoradiograph of Northern blots hybridized with the S (lanes 4 and 5) or the M probe (lane 6). Lane 4 - 9938; Lane 5 - Ca1; Lane 6 - 9938. S signal sizes in lanes 4 and 5 are 8.48 and 6.14 kb (GEL program estimates).

RNA Dot Blots. Nitrocellulose blots were prepared with samples from 9938 and Ca1 total RNA (as above), and 9938 mRNA enriched samples collected from oligo-(dT) columns. Dot blots were probed with actin, L, M, or S fragments (Figures 23, 24). MCID analyzed signal densities from RNA spots are listed to indicate the signal strengths. The actin and L probes were hybridized to total RNA, mRNA samples, and 60 pg spots of the L and actin DNA to serve as non-specific controls for the opposite probe (Figure 23). Positive L hybridization signals to the 50ng mRNA spot, the 5 - 20 µg 9938 total RNA spots, and the 60 pg L DNA spot were observed, but non-specific hybridization of the L fragment to the 60 pg actin DNA spot was not seen (Panel A). The actin probe hybridized to mRNA (25 and 50 ng), total 9938 RNA spots (5 - 20 µg) and to actin DNA (Panel B). No non-specific hybridization to heterologous DNA occurred (Panel B, 60 pg L DNA spot).



C

Dots		OD Values[1] L	Actin
µg total RNA	0	0.19	0.21
	5	0.21	0.33
	10	0.22	0.44
	20	0.25	0.60
60pg L DNA		0.45	0.19
60pg Actin		0.21	0.28
ng mRNA	25	0.19	0.24
	50	0.21	0.45

Figure 23. Northern dot blots probed with the L fragment and actin. Panel A and B: Sample dots were applied in the same order for both panels. From left to right in Row 1: Yeast form 9938 RNA in 0, 5, 10, and 20 µg amounts, ϕ, 60 pg L DNA, and 60 pg actin insert DNA. Row 2: ϕ, and mRNA enriched samples dotted in 25 and 50 ng amounts (quantified by DNA Dipstick Kit). Panel C: OD values from scanning panel A and B autoradiographs. [1] MCID system was calibrated to standard OD units.

Another series of dot blots including DNAse treated RNA samples which were from CsCl (9938Y-Tx) and acidic-phenol extraction (Comp-Tx) methods (methods section), total RNA samples, and negative control tRNA dots were probed with actin, L, M, and S DNA fragments (Figure 24). The MCID OD values for the 20 µg tRNA and 60 pg ura3 DNA dots for each probe are not above the "blank" OD values indicating that the probes do not non-specifically bind to the sample positions. RNA samples in the last two rows are from independent cultures and different from the CsCl RNA samples included in Figure 23.

All four probes produced signals with the DNAse treated RNA samples (from either the CsCl or phenol method) seen in the 9938Y-Tx rows in Figure 24. The M and S probes produced weak signals with the 5µg 9938Y-Tx sample. CsCl method RNA samples (bottom 3 rows, Figure 24) were positive for actin and L (like Figure 23) and the M and S probes were also positive.

Again, the actin probe produced a stronger signal with the pseudohyphal RNA sample than the yeast sample for 9938 as noted for Figure 21. Though the dot blots were initiated to investigate qualitative RNA expression for the L, M and S fragment, the S probe indicates a stronger signal for Ca1 yeast and 9938 pseudohypae than for 9938 yeast form. For both actin and S expression, the difference may be associated with just the 9938 isolate and further quantitation of RNA levels in yeast, pseudohyphae, and mycelia of various isolates would be necessary to establish actual difference and significance of the data.

The formaldehyde gel and dot blot Northern hybridization data indicate that sequences from L, M, and S are expressed at the RNA level.

The probes bound specifically and did not cross-hybridize with heterologous RNA or DNA spots. The L and actin probe hybridized with mRNA samples, DNAse treated RNA, and CsCl method RNA from two separate RNA extractions. The S and M fragment probes hybridized to DNAse treated RNA and total RNA samples. The presence of open reading frames found in the partial sequence information for L, S, and M fragments (presented below) support the Northern blot results.

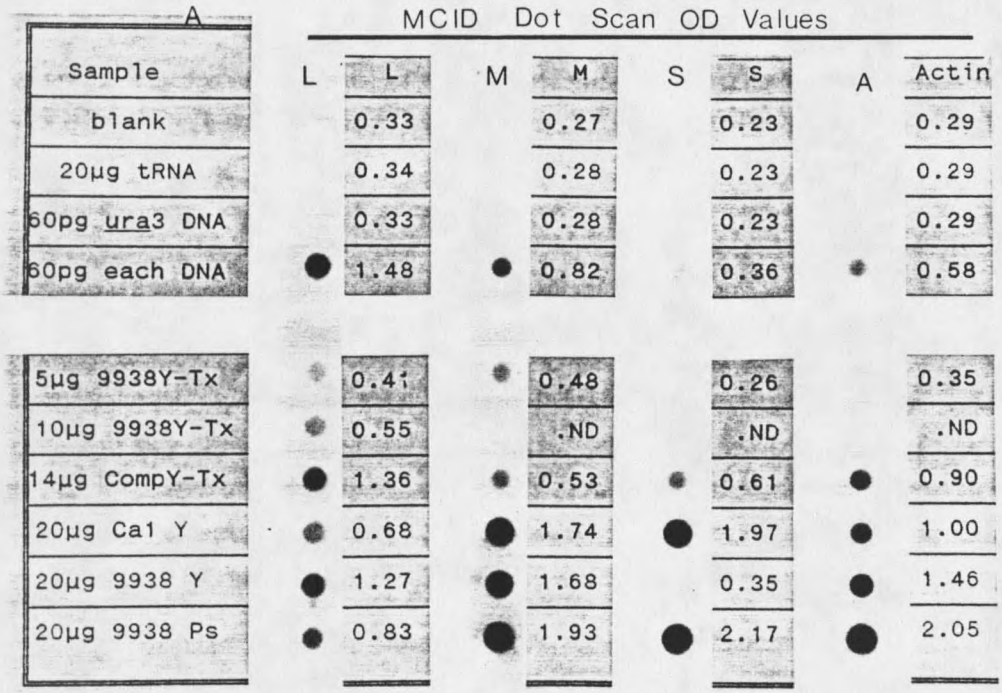


Figure 24. Northern dot blots probed with L, M, S, and actin. Total and DNAse-treated (Y-Tx label) yeast RNA samples were applied in the same order for each blot as follows: blank (0µg) or background; 20µg tRNA; 60 pg ura3 gene DNA; 60 pg each DNA (L, M, and S insert DNA and actin gene DNA); 5µg 9938Y-Tx; 10µg 9938Y-Tx for L blot only or not determined (ND) for the M, S, and actin; 14µg CompY-Tx (9938 RNA by phenol method); 20µg Ca1 Y (yeast form, CsCl); 20µg 9938 Y (yeast form, CsCl); 20µg 9938 Ps (pseudohyphal form, CsCl). MCID OD values for all four blots were obtained in the same scan with the system calibrated to standard OD units. All strips were exposed for 48 h, -70° C, with intensifying screen to the same film piece. The blank dot scan indicates the background value for each strip. All Candida RNA target spots for each probe are positive indicating that L, M, and S sequences are expressed.

Subcloning of pHpaCa7 Fragments

Plasmid miniprep DNA samples of the XL1-Blue (E. coli) transformants were digested with PstI and sized on 1.5% TAE agarose to identify pHpaCa7 insert pieces in pBluescript II vectors (Figure 25). Compared to PstI-digested vector only (lane 9), plasmids in lanes 2, 3, and 5 contained an insert of approximately 1.8 kbp (arrow), the expected size of the small PstI piece of pHpaCa7 (Figure 8). Lanes 7 and 8 showed a large insert of 7.5 - 8 kbp (arrow, GEL program estimates) corresponding to the other PstI piece of pHpaCa7. Plasmids from lane 5 (pBSPst+12) and lane 7 (pBSPst+18) were selected for sequencing.

Lanes 10 - 13 of Figure 25 contain MspI digested plasmids generated by J. Cutler, T. Kanbe, and L. LaTray when MspI fragments of pHpaCa7 were subcloned. Lanes 10 - 13 indicated possible inserts of the 1.6 kbp M fragment (lanes 10, 12, and 13) and the 0.8 kbp S fragment (lane 11). The plasmids pBSMsp-S9 (lane 11) and pBSMsp-M22 (lane 12) were included in sequencing reactions.

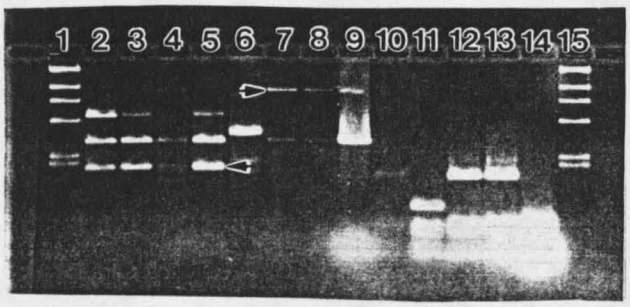


Figure 25. pBluescript II SK(+/-) Subclones of pHpaCa7. Vector and recombinant plasmid miniprep samples were digested with PstI (lanes 2 - 9, vector was pBluescript II SK+) or MspI (lanes 10 - 14, vector was pBluescript II SK-) and separated on 1.5% TAE agarose. Lanes 1 and 15 contain Lambda HindIII marker DNA, lanes 9 and 14 are vector only controls. Arrows next to lane 5 and 7 indicate the small and large PstI pieces from pHpaCa7. Lanes 11 and 12 were also included in sequencing reactions.

None of the MspI subclones indicated an insert as large as the 3.8 kbp L fragment, so J. Cutler and L. LaTray size selected the entire L fragment, digested it with ClaI (see Figure 8), and subcloned the resulting 2.2 kbp and 1.6 kbp pieces into pBluescript II SK (-). Two plasmids, pMCL-4 and pMCS-1, were identified with inserts of appropriate size and were included in sequencing. During subcloning, they observed a low frequency of white transformants that prompted a miniprep inspection of "background" blue colonies. ClaI digestion of miniprep samples indicated that some of the functional ß-galactosidase colonies contained plasmid inserts of 2.2 and 0.8 kbp in size. Sequence information showed that the 2.2 kbp ClaI/MspI L fragment was ligated in the opposite orientation as the 2.2 kbp insert in pMCL-4 (from a white colony), and the 0.8 kbp insert sequence was rearranged pBluescript vector (data not shown).

Dideoxy Sequencing

Portions of the C. albicans L, M, and S fragment sequences were obtained and are reported in Figures 26 - 34. Short and long gel run information was compared by the Align program to obtain the longest continuous sequence for each segment. In the event of nucleotide differences, the autoradiographs were reinspected to confirm or represent ambiguities by N's. In each figure, vector bases read from the beginning of primed sequences are shown in lower case letters indicating the confirmed ligation site of pHpaCa7 and the various subclones. C. albicans bases are shown in upper case letters and are the only numbered bases (Figures 26 - 32). Nucleotides reported here are from sequencing one strand and are subject to confirmation by

complementary strand sequencing.

A Protylze program (Protylze Predictor Version 3.0, Scientific & Educational Software) which scans all three reading frames in both strands was used to identify open reading frames (ORFs) from the Candida sequences. The ORF regions are noted with bold-face start codons ATG or CAT for the complementary strand. ORF regions (≥5 amino acids) are listed below each sequence inside the figures. The Candida nucleotide information was also used to query the GenBank database by FastA sequence similarity search (311). An arbitrary criterion of >68% homology over ≥45 nucleotide length was chosen for mentioning specific cases of GenBank homologies.

pHpaCa7 Sequence. Partial sequence information for the S and M fragments was obtained directly by sequencing denatured pHpaCa7 with pBR322 primers: a clockwise (cw) primer from the 5' EcoRI side (S fragment) of the insert and counterclockwise (ccw) primer from the 3' HindIII side (M fragment) (see Figure 8). Open reading frames (ORF's) lengths in amino acids (aa) are noted with the start codon (ATG or CAT for complementary strand) in bold-face print and a (c) indicating that the reading frame was on the complementary strand. CAG repeats (***) and RGYCAG (---***) are marked.

The 330 Candida nucleotides of the 0.79 kbp S fragment (Figure 26) contain CAG trinucleotides (*** marking) which were repeated 22 times. Thirteen repeats were part of a hexanucleotide repeat RGYCAG (---*** marking) where R = A or G and Y = C or T with G and C most common. Nine of the hexanucleotide repeats were tandemly arranged. Three open reading frames (ORF's) longer than 5 amino acids (aa) were indicated for

the partial S sequence. Table 5 contains the best FastA scores indicating that the CAG repeat region (approx. 135 - 227 bp) produced the majority of FastA matches.

```
agcttatcatCGGTTGCCAACGCTTGTACCATTCTCTAGTACCAACTTAC  40
ATCCTGGGGACTGGTCTACTGCCACCCTTGGCCAGAAAATCTGGGCTGGC  90
                                ---***

GGGAACAAGGGCTGGGGCTGAGATCAAGCTTGGGCCAGAGCAGAGCCAGA 140
                                   ---***  ***---***-

GCCAGAGCCAGAGCCAGAGCCAGAGCCAGAGCCAGGGTCAGGGCCAGGGC 190
--***---***---***---***---***---***---***---***  *

AGACAGACCAGGGTCAGGGCCAGGGTCAATGGCTGACGTCCTAAGCTCAA 240
** ***  ***---***---***

GGGAATGGCCAATCCAGAGCAGGTCTAAGCCAAGCCACAGTATGCTATCA 290
              ***  ***               ***        **

GTACAAGTAGCGGCTAGTACCTGCCACTTACAGAGCTCAA
*                             ***
ORF's: 22-->2 (c) (7 aa)
       217-->328  (37 aa)
       243-->264  (7 aa)
```

Figure 26. Nucleotide sequence of pHpaCa7 with EcoRI cw primer into the S fragment. Numbers reference the C. albicans nucleotides.

Table 5. FastA search of partial S fragment sequence.

S Region	Percent Homology	Basepair Overlap	GenBank sequence
132-208 (c)[1]	83.8	74	Rat chondroitin sulfate proteoglycan core protein mRNA
132-208	76.3	76	Maize opaque(02) mRNA, zein deposition regulation (leucine zipper)
131-180	77.6	49	Hu. Sp-B pulmonary surfactant protein

Table 5. FastA search of partial S fragment sequence, continued.

131-198	76.1	67	Hu. cyclin B mRNA, 3'end
118-198	71.3	80	Hu. ryanodine receptor mRNA (Ca^{+2} release channel of muscle)
27-178 (c)	68.9	61	Hu. episialin (varB) mRNA (mucin)
147-190 (c)	79.1	48	Hu. secretory granule proteoglycan core gene, exon 3
134-208	70.1	74	Hu. Platelet-derived growth factor receptor mRNA
125-218	72.3	83	Mouse hexamer repeat sequence (17)
129-180	82.4	51	Mouse Sp5 mRNA, period repeat
129-188	76.3	59	Spinacea oleracea ribosomal protein L21 mRNA
135-199	76.6	64	Hu. HMG-I protein isoform mRNA
115-208	68.8	93	Plasmodium berghei circum-sporozoite (CS) protein gene
139-247 (c)	71.3	108	Mouse Ig Kappa unproductively rearranged V
116-238	70.5	122	Mouse MHC Class I H2(TLA)-Qa-2,3 pseudogene transplantation antigen

[1](c) indicates that the numbered region of homology was from the complementary strand

The M fragment sequence of 540 nucleotides (of 1.6 kbp total) was 63% A-T in composition with a CCGG site 95 bp from the ligation site (Figure 27). If correct, the CCGG site should have been cut by MspI in Figure 3 to give a fragment approximately 230 bp long (part pBR322 and part C. albicans DNA). Then the other portion of the M fragment should not have hybridized with the pBR322 probe unless: a.) the digest was incomplete or the CCGG site at bp 95 was protected, or b.) pBR322 sequences were present someplace else along the fragment. The Align

program results indicated that nucleotides from regions 1 - 95 or 96 - 540 in Figure 27 did not match any region of pBR322 sequences when specifically checked. Three separate Southern blots of pHpaCa7 MspI fragments (Figure 7 plus two blots of MspI digested total pHpaCa7) probed with labeled pBR322 resulted in an M fragment signal, asserting that the first possibility would be unlikely. Evidence that pBR322 recombined with M fragment regions during pBluescript subcloning was observed (presented later in Figure 33 and 34), but recombination in the original M region of pHpaCa7 was not investigated. As mentioned for Figure 7, possible smaller fragments of pHpaCa7 (<150 bp) digested with MspI were not studied.

Compression of bands accounted for an unreadable region of the M fragment (represented by N's in Figure 27) which remained unresolved in all gels of ccw HindIII primed reactions including dITP reactions. Nine ORF's longer than 11 amino acids are listed for the partial M sequence (Figure 27). Region 31-171 (complementary strand "(c)") was 74% homologous to rDNA genes and flanking regions of S. cerevisiae and S. carlsbergensis according to FastA similarity search (311). Region 208-253 (c) was 73.3% similar to S. cerevisiae MIP1 gene for a mtDNA polymerase catalytic subunit; and region 57-109 (c) was 80.8% homologous to Mucor racemosus 25s rDNA region.

pHpaCa7 Subclone Sequences. pBluescript subclones, annealed with the T7 forward primer (FP) or the M13 reverse primer (RP) for the vector multiple cloning site (MCS), provided nucleotide information for portions of the internal regions of the pHpaCa7 insert. Subclone pBSPst+12 contained approximately 800 bp at the 5' end of the L fragment

and all of the S fragment (5' PstI piece, Figure 8 map). RP sequencing of pBSPst+12 confirmed restriction digest indications that the pBR322 end was ligated to the SacI side of the MCS (data not shown). Figure 28 contains FP nucleotide information from the Candida end in pBSPst+12. Again, pBluescript bases are shown in lower case letters. The 186 bp of Candida DNA are approximately 750 bp downstream of the 5' end of the L fragment. FastA search results indicated only one 45 bp region (bp 124-169) with 73% homology to S. cerevisiae mtDNA oxi3 and cytochrome loci. Two ORF's are indicated in Figure 28.

```
aagcttatcgGATCGCCAGAGGGCGGAATGCTAGAACGTGGAGTTTGACC  40
TCCAGCTCCGCTTCATTGAATAAGTAAAAAACTATAGAGGTAGTGGTATT  90
TCACCGGTGATGGAAATCCACCAATGTATAATGACGACGCCATTGACTTT 140
ATTCAGAATATACCTGGAATCAATGCAGTTAATTACCACATTTTGCATTG 190
CCAACAGGTTAAGAATATTGAAGAATTCGGTATCTTTATCCAAAGATAAG 240
TTTATATCGATATTAGGTGATGAGAATGGCAAGAAAGCATATATTTTTTG 290
AATAGGAAAATTAAATAGCTGTGACAACGGAGAAGCAACGGCCTCACTAT 340
TACTGCTGATTTATTGTGTGTCTATTTCGCTTCCACAGCCATCTTCTTGT 390
TTNNNNNNNTTAGCTCAATAGTTATATATATGCACTATTCAGAGAGATAT 440
AGTAGTATAGACTGAACGAATAACGTATCATGACATACTTTGTTCGTTAT 490
TTATATCTGAGCTTAGATTCTTCTTGATACATGTCATTAT

ORF's:  110-->299 (63 aa)     124-->247 (41aa)
        188-->74 (c) (38aa)   133-->55 (c) (26 aa)
        527-->470 (c) (19aa)  471-->414 (c) (19aa)
        470-->515 (15 aa)     280-->244 (12 aa)
        260-->293 (11 aa)
```

Figure 27. Nucleotide sequence of pHpaCa7 with HindIII ccw primer into the M fragment. Numbers reference C. albicans nucleotides.

Sequencing reactions with denatured pBSPst+18 (FP or RP) did not produce readable nucleotide sequence possibly because of the large plasmid size (approximately 11 kbp).

```
attcctgcagGGATGTCAGCCTCGATAACGAAACAGAACGAGTAGTACAA   40
GCTGTTATTACAAGAAGTCAACCCATTTTGCTTTATGCTTTACTATTATC   90
TAGGTTTGGAAAAGTGTGAACTTAGTATACAGCCAATAACAGAATTAATA  140
AAAATACGATTCCTTATATTATGTAAGTACAAGGATACTCTCAG        184

ORF's:  75-->183 (36 aa)
         3-->30  (10 aa)
```

Figure 28. Nucleotide sequence of pBSPst+12 with FP. Lower case letters are pBluescript bases and numbers refer to Candida nucleotides.

Four MspI subclones of pHpaCa7 (J. Cutler, T. Kanbe, and L. LaTray) were included in the sequencing reactions. Partial nucleotide sequences from the two ClaI by L subclones (pMCL-4 and pMCS-1) are shown in Figures 29 - 32 with pBluescript vector nucleotides noted in lower case. The insert orientations of the two L subclones were not determined with regard to pHpaCa7 5'-->3' direction (Figure 8). The dGTP reactions produced more readable sequence from both pMCL-4 and pMCS-1 plasmids; dITP results suggested Sequenase stall points which the supplier (USB) stated as possible for sequences with exceptional secondary structure (data not shown).

For the 2.2 kbp insert in pMCL-4, the larger ClaI L subfragment, 382 bp where obtained in forward priming reactions (Figure 29) and 308 bp by reverse priming (Figure 30). FastA search of pMCL-4 FP and RP sequences indicated that region 32 - 116 of the RP sequence was 72.6% homologous to chicken prostaglandin G/H synthase mRNA. Five ORF regions were identified in the FP and RP sequences of pMCL-4.

```
gacggtatcgGGGCTAGTACTCTGGCCCACTCTACAAGAGGCTCAAGGGT  40
GTTGGGTAGCAGTCAATTGTAATGGCTGTGCCAGGTTCACCACTGGCAAC  90
ATGATTGGTCCTAGTGCCTAGCCCAGCTTTGAGGCCAATACAACTTCTGG 140
CTAACCAACCAGAGTTTATGGCAGGCCAGCTTTCAATTTGGTCCCAAAAC 190
TGGTTGAAATATTGCTGTCAACGATGTTCCTCGGCCAAAGTTAGGGCCGT 240
TAAAAGGAACAGGACCAATATTGTTTCCGAAGCCAGTTCAAATTTCTTTG 290
CTGTGTTGGTAACTTGACTTGTCTCGGATATACCAATGAAGGCAGAGATC 340
AGACAGTCTAAAGTCGCTACTGTTACAGATGCTGACTGGCAA

ORF's:  158-->305 (49 aa)    326-->383 (19 aa)
        92-->35 (c) (19 aa)   62-->92  (10 aa)
```

Figure 29. Nucleotide sequence of pMCL-4 with FP.

```
aagcttatcgATTTATCATTTGCTTTAAAAATTGCCAATACTCCCTATTT  40
TTCTGACGTTTTCAGTTTGATTGGCATTATATTATTTCCTCGAATACTTT  90
CAATTTTTAATAATTACAAATTTTTTAATTTGATTGTATTATCCTTCAAT 140
AAAATGATCTGGAACTTGGTTGGATTGATCTTTTTCTTTTTCACATTGAT 190
CAGTGGATTTTATTTCAGTTTTTAACCTAAGTATTAACCAAGCCAAGGTG 240
AATATTTTGATATGGTCAAATATTTTGGATTCACTCCTCAGTATGGAATA 290
TGGAATACACTGTGGAATAC

ORF's:  144-->213 (23 aa)
```

Figure 30. Nucleotide sequence of pMCL-4 with RP.

Sequences from the smaller ClaI by L subclone, pMCS-1 (approximately 1.6 kbp insert), are shown in Figures 31 and 32. In FP sequencing 308 bp were read (Figure 31) and for the RP reactions, 235 bp were obtained including an unresolved GC compression region (N's in Figure 32). FastA search revealed four small pMCS-1 RP regions of homology: a.) bp 182-230 are 72.9% homologous to Thermoproteus tenax virus 1, viral protein T; b.) bp 4-49 (c) show 73.3% homology to an African Green Monkey origin of replication; c.) bp 9-60 (c) are 72.5% homologous to bovine mitochondrial ATP synthase gamma subunit; and d.) bp 15-62 (c) show 70.2% homology to Human small ribonucleo-protein (snRP8). FastA search results for the pMCS-1 FP Candida sequence

included no homologies within the arbitrary bounds (≥45 bp with >68% homology).

```
gacggtatcgATTAAGAAAATCAAGCAAAAATCAATATAATTCCAAATTA  40
TAGTTCTCACAAATTGCCATTCTATATTGTACAATTTTGTAATTAATTCA  90
ATATTAAAACTTAAATTAATAATCCAAAATAAAGTCCCAATTCCCATAGG 140
GTTGCCCAAAGATCAACAGAAAGTAAAAGATCAGTAGCATTATCAATTTG 190
AAATTTATCACTATGGATTGATATTTTGGCACGGTGTTTGCCCGTTTAAT 240
TATTAGTTATATGATATCTCTAATTGGTATCTGTACACAGTCAGCTTCAT 290
TACCTTTCATCGTCAGTATA

ORF's:  135-->1 (c) (45 aa)   178-->106 (c) (24 aa)
        249-->309 (20 aa)     298-->240 (c) (17 aa)
        288-->243 (c) (15aa)  58-->22 (c) (12 aa)
```

Figure 31. Nucleotide sequence of pMCS-1 with FP.

```
aagcttatcgATATTAGGTGATGAGAATGGCAAGAAAGCATATAATTTTT  40
TGAATAGGAAAATTAAATAGCTGTGACAAGGGAGAAGGAAGGGGCTCACT  90
ATTACTGCTGATTTATTGTGTGTCTATTTCGTTTCACAGCCATCTTCTNN 140
NNNNNNNGGTTGCTTTACGCATTGTTTGTTACCTTTAGGCTCAAATAGTT 190
AATATATATGCACCTATTCAAGGAGAGATATAGTAGTATAGGACATGA

ORF's:  198-->237 (13 aa)    131-->92 (c) (13 aa)
        160-->124 (c) (12aa)  11-->41 (10 aa)
        234-->204 (c) (10aa)  28-->1 (c) (9 aa)
```

Figure 32. Nucleotide sequence of pMCS-1 primed with RP.

Sequencing of single-stranded templates from pMCL-4 and pMCS-1 did confirm FP sequence but did not provide longer nucleotide information than that derived from priming denatured plasmid templates (data not shown). One MspI subclone, pBSMsp-S9 in lane 11 of Figure 21, contained a pBR322 vector fragment (nucleotides 2350 - 2600) instead of the S fragment (data not shown). Another pBluescript subclone, pBSMsp-M22, contained rearrangements of pBR322 and the M fragment (explained below) revealing additional C. albicans nucleotides (Figures 33 and 34).

The Align program was used to compare the FP and RP sequences to pBR322, pBluescript, and to the ccw primed sequence from original pHpaCa7 (Figure 28). In Figures 33 and 34, pBluescript nucleotides are in boldface lower case, pBR322 in lower case, and Candida bases are in uppercase letters. A diagram (Figure 35) was prepared to illustrate the rearrangement of sequences for reference. The forward primed reactions of pBSMsp-M22 indicated that the pBluescript multiple cloning site (MCS) flank was attached to an inverted piece of the Candida M region (15 - 95 in Figure 35) beginning with the CG of the CCGG site from position 95 in Figure 28. Next, bases corresponding to Candida region 14-1 (marked with +'s) through pBR322 bp 84 (Figure 35) were found in the pBSMsp-M22 subclone (Figure 33). The Candida nucleotides which followed (numbered Ca1-Ca226 in Figure 33) did not match any of the M region in Figure 28 or any pBR322 or pBluescript nucleotides. On the other end of the pBSMsp-M22 subclone, the RP nucleotide information (Figure 34) indicated that the pBR322 region 76 - 161 (Figure 35) was attached to the MCS flank of pBluescript. Again, the Candida nucleotides which followed (Figure 34 numbered Ca1 - Ca176) did not match any of the M region revealed from pHpaCa7 by ccw HindIII priming (Figure 28) or vector sequences (pBR322 or pBluescript). pBR322 bp 76 - 84 nucleotides (GGCACCGTG, ^ marking in Figures 33 and 34) were duplicated during the rearrangement of the M fragment and appeared in sequences of FP and RP reactions of pBSMsp-M22. ORF regions are listed for these M fragment regions (Figures 33 and 34).

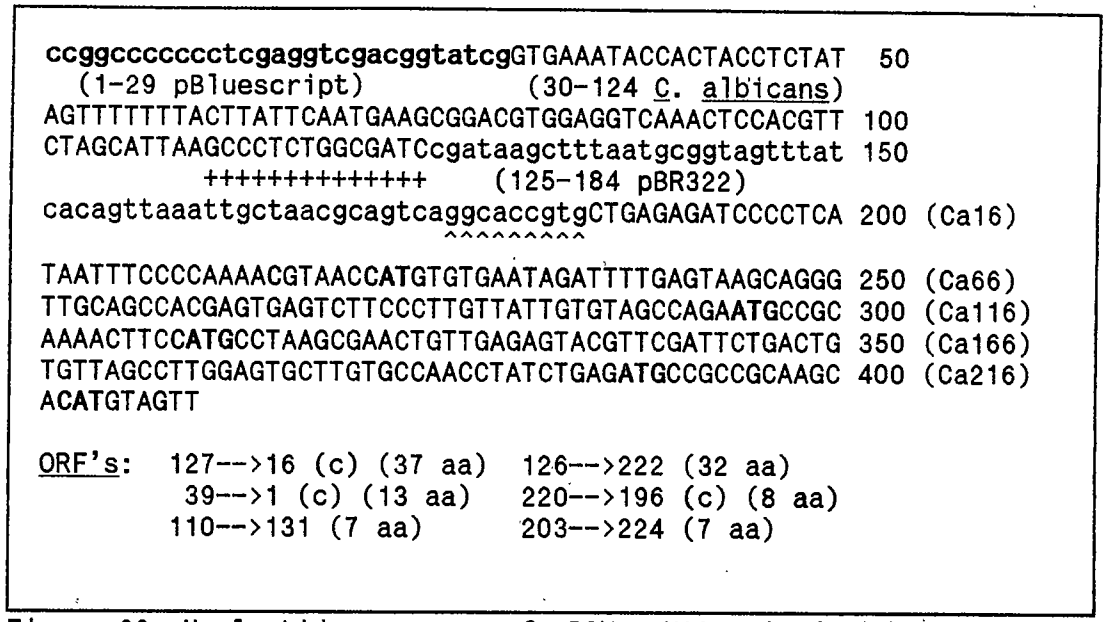


Figure 33. Nucleotide sequence of pBSMsp-M22 primed with FP. C. albicans nucleotides are upper case, pBluescript bases are boldface letters, and pBR322 bases are lower case letters.

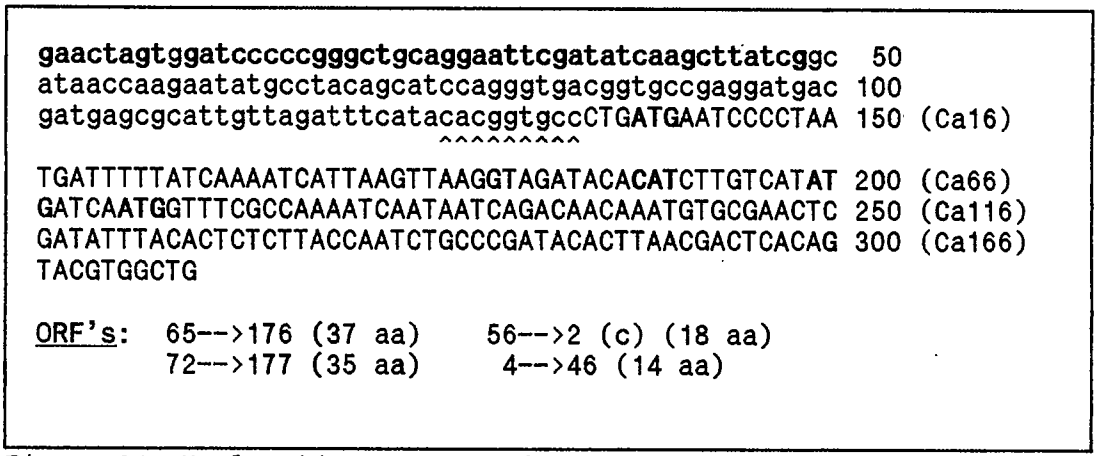


Figure 34. Nucleotide sequence of pBSMsp-M22 primed with RP. C. albicans nucleotides are upper case, pBluescript are boldface, and pBR322 bases are lower case letters.

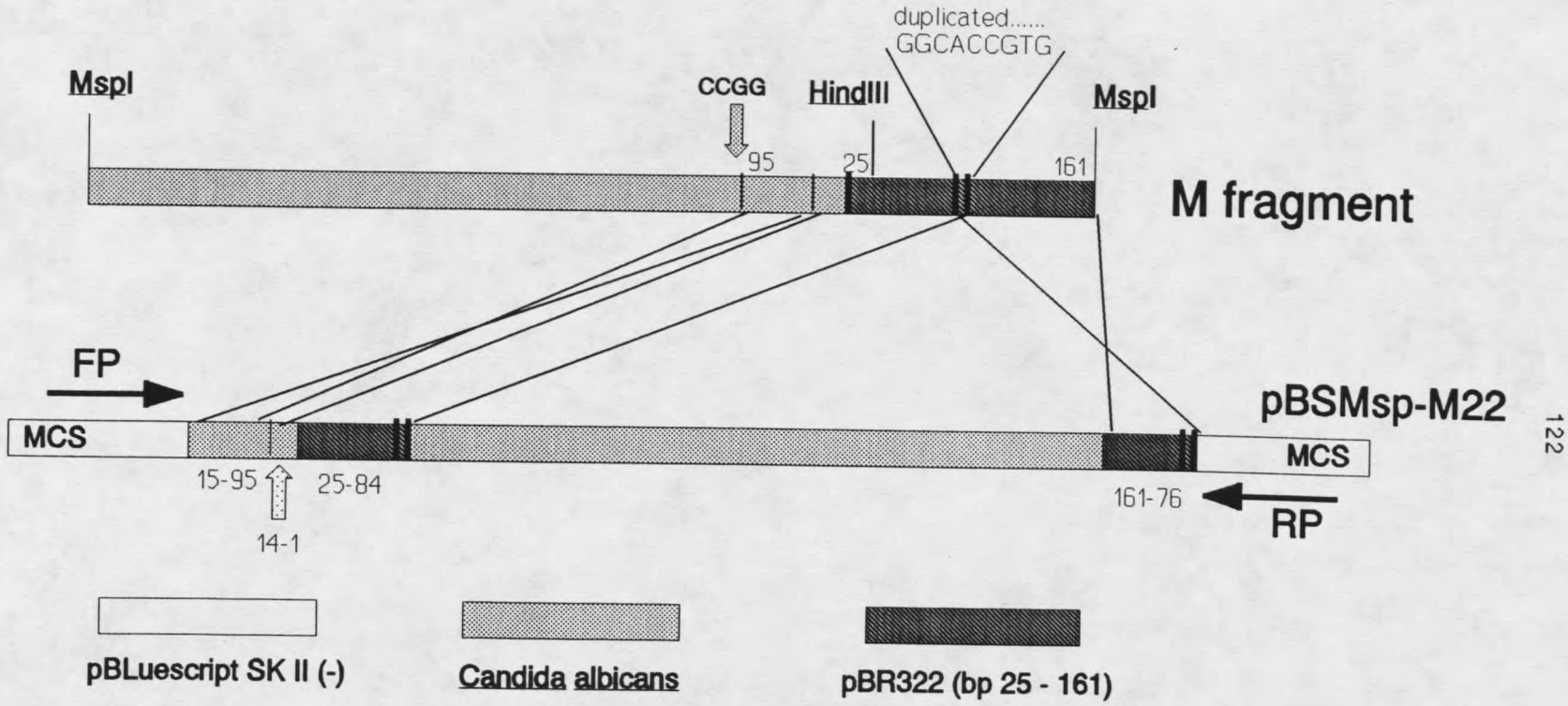


Figure 35. Rearranged M fragment in pBSMsp-M22 subclone.

DISCUSSION

The principal aims were to extract high quality DNA from C. albicans and characterize a DNA fragment with indicated species-specific hybridization. For the first research goal, mechanical and enzymatic methods of DNA release were applied to cells grown under common culturing conditions. C. albicans DNA analysis with methylation sensitive restriction enzymes suggested repetitive sequences in the genome. For the second goal, the DNA fragment was characterized with respect to extended DNA survey, restriction mapping, copy number, RFLP patterns, yeast and mycelial forms, nuclear or mtDNA origin, evidence of transcription, and partial sequencing. Amid pursuit of the second research goal, two other cloned fragments were identified and partially characterized. Discussion of the principal and associated results are presented below.

DNA Extraction from C. albicans

Polysaccharide-protein polymers in fungal cell walls present tough barriers for the extraction of DNA. Methods for releasing DNA apply various mechanical or enzymatic tools to handle the problematic cell wall. Physical forces chosen for mechanical breakage of cells must be enough to rupture the cell wall, but not so much as to cause excessive shearing of DNA molecules. Enzymatic digestion of cell walls has been done with various enzymes of assorted degrees of purity, but different

cell wall compositions among fungi prevent defining universal digestion conditions. During digestion, the resulting fungal spheroplasts must be prevented from undergoing osmotic lysis. The concentration and type of sugar, alcohol or inorganic salt used as an osmotic stabilizer must be determined for each type of fungus (287). Dealing with the cell wall of C. albicans presents these same difficulties in obtaining high quality DNA samples suitable for molecular genetic analysis.

Three different extraction methods were compared for the capacity to yield high quality DNA from C. albicans as judged by two criteria: isolation of high molecular weight fragments; and, susceptibility of DNA samples to digestion with restriction enzymes. Disruption of the cell wall by Ribi Cell fractionation, autolysis, or spheroplasting was gentle enough to provide subsequent DNA fragments which averaged over 30 kbp in size and could be digested by all restriction endonucleases tested [Figure 1 and reference (298)]. DNA samples obtained by any of the three methods were suitable for many kinds of molecular analyses, including RFLP comparisons (Figures 1, 9, and 15), general cloning (99), and construction of genomic libraries in plasmid or phage vectors.

Ribi Cell fractionation, autolysis, and spheroplast DNA extraction methods gave approximately the same yield of DNA at 2µg per 1 x 10^7 cells, which represents 50% recovery based on the estimate of 40 fg DNA/diploid cell (239). The yield value was generally used to determine culture volumes necessary for obtaining required amounts of DNA for a particular application. Multiple, small-scale samples were more easily processed by spheroplast methods, whereas large volume extractions with a minimum breakage volume of 10 ml were done by Ribi Cell fractionation.

The autolysis method could be used for small and large extractions, but the entire procedure takes longer (298). Other specific observations on these methods are discussed below.

DNA Extraction from Spheroplasts

Spheroplast DNA extraction has been used frequently for the preparation of yeast DNA, especially from Saccharomyces, but cell wall composition differs among yeast genera and species, and requires adjustment of enzymes or culture age for efficient spheroplast production (28,287,289-292). Previous Candida research indicated that: DNA can be obtained from C. albicans spheroplasts which were produced by digesting whole cells with a mixture of helicase and chitinase after a proteinase pre-digestion step (293); a low concentration of Zymolyase enzyme (eg., 0.1 mg/ml or 2 Units/ml) was effective at removing C. albicans wall components (52,319,320); and, a higher concentration of Zymolyase (20 U per 1 x 10^8 cells) was used to prepare log phase C. albicans cells (grown in defined media) for spheroplast fusion experiments (163,224,321).

Zymolyase was chosen in this work for spheroplast production because of the absence of DNAse and RNAse activities. Enzymatic activities of Zymolyase include ß-1,3-glucanases (ß-1,3-glucan laminaripentaohydrolase and ß-1,3-glucanase), protease, mannanase, and minute amounts of amylase, xylanase, and phosphatase, but no ß-1,6-glucanase activity (technical sheet for Zymolyase 20T, ICN ImmunoBiologicals).

C. albicans susceptibility to Zymolyase. The studies reported here indicate that the susceptibility of C. albicans cells to spheroplast

formation by Zymolyase 20T (80 U/g cells) varies with growth phase and culture medium. C. albicans yeast cells grown in GYEP exhibited isolate-associated differences and stationary phase-associated reduction of spheroplast efficiency with Zymolyase (Figure 4). Stationary phase reduction in spheroplast efficiency has been noted by others (290,293).

Log phase and stationary phase C. albicans yeast cells grown in mLee medium were generally more susceptible to Zymolyase digestion than cells grown in GYEP. For example, strain 9938 yielded 75 - 85% spheroplasts from GYEP log and stationary phase cells (Figure 2); whereas from mLee, 9938 yielded greater than 95% spheroplasts from all growth phases. Isolate Ca1 (Figure 2, 80% spheroplasts from GYEP stationary phase), CaO, and Ca142, all yielded 98 - 100% spheroplasts from mLee stationary phase cells. These results suggest differences for mLee-grown verses GYEP-grown cell wall structure relevant to the enzymatic activities present in Zymolyase. It may be possible that mLee-grown cells and log-phase cells, in general, have fewer polymers that are resistant to Zymolyase digestion (see below for further discussion).

Cell Wall Variability. The variable characteristics of Candida cell wall structure have been extensively documented [(188,322,323), and reviewed in (8,22,24,51,63)] and are likely related to the observed growth phase differences in spheroplasting efficiency. Growth conditions, growth stage, and morphogenic form of C. albicans cells are factors which influence the number and relative composition of wall layers (51,52). Components of C. albicans cell wall [reviewed in (22)] include: mannans, that make up 15 - 23% of yeast cell wall dry weight; ß-1,3-D-glucans and ß-1,6-D-glucans at 47 - 60% of cell wall dry weight;

proteins, that account for 6 - 25%; lipids for 1 - 7%; and chitin for 0.6 - 9% of cell wall dry weight [reviewed in (22)]. Chitin polymers are linked to glucans in the inner wall layers, exhibit higher concentrations in yeast bud scars, and display 3 times more chitin in hyphal cell walls than yeast forms (51,164,294,322,324).

Mannoproteins are distributed throughout the layers of the cell wall with the greatest concentration at the surface, where they function as primary antigenic molecules of C. albicans [reviewed in (22,51)]. The amount of mannoprotein varies with growth medium and age of the culture, and the mannoproteins are removed easily from cells (325-327). Mannoprotein contribution to Zymolyase resistance would likely be minimal because of their predominant surface location and easy extraction, even though the mLee- versus GYEP- grown cells and various growth phases would have different amounts of mannoprotein.

The ß-glucan polymers, especially ß-1,3-glucans, are thought to be primarily responsible for the rigid cell wall framework for both C. albicans and S. cerevisiae (28,328). Regenerating spheroplasts make ß-1,3-glucan polymers during initial reconstruction of the wall (194,294, 329). Three types of chemically extracted glucan fractions have been identified from the C. albicans cell wall: alkali-soluble glucans containing 70% ß-1,6-glucan (unbranched) which make up <10% of the total cell wall material; acid-soluble, highly branched glucans containing 67 - 77% ß-1,6 linked residues; and an alkali- and acid-insoluble, highly branched complex with 30 - 40% ß-1,3-glucan and 43 - 53% ß-1,6-glucan (24,51). Branch point residues between ß-1,3- and ß-1,6-glucans ranged between 5% and 13% for the various glucan fractions (24).

The relative amounts of ß-1,3- and ß-1,6-glucans in C. albicans yeast cells grown in two different complex media are known to vary (322), but no studies have been reported which examined the cell wall glucan components for defined verses complex media. The different susceptibility that Candida cells displayed to Zymolyase (ß-1,6-glucanase deficient) may be due to the presence of more branched ß-1,6-glucan polymers or ß-1,3- to ß-1,6-glucan branch points contributing to resistant cell wall structures.

Zymolyase Induced Hyphal Lysis. In experiments reported here, Zymolyase 20T (80 U/g cells) caused lysis of 3 - 4 h germinated cells in the same osmotic conditions used for yeast form spheroplasts. Lysis of the hyphal cells during Zymolyase incubation prohibited DNA extraction with the normal spheroplast procedure. Hyphal cell walls have thinner ultrastructure than yeast walls as evidenced by transmission electron micrographs (323,330), but they have little difference in the overall proportion of glucan and mannan compared to yeast cell walls (322). Strikingly different ß-1,3- to ß-1,6-glucan proportions were observed for the insoluble glucan fraction in germ-tube forming cells, which had 67% ß-1,3-glucan and only 14% ß-1,6-glucan, compared to 30 - 40% ß-1,3-glucan and 43-53% ß-1,6-glucan in yeast and hyphal form walls (328,331). The 3 - 4 h germinated cells in these studies would be considered germ-tube forming cells, prior to hyphal septation. Other investigators have obtained spheroplasts from 4 - 24 h Candida hyphae (grown in Lee medium) by using helicase and chitinase (293) or ß-1,3-glucanase, protease, and chitinase, and they indicated that addition of chitinase was crucial for producing spheroplasts from hyphae (294,332). Zymolyase plus chitinase

was not tested in this work, nor were other osmotic conditions to promote the stability of C. albicans hyphae. The basis for hyphal-spheroplast lysis under these conditions was not determined, but may be associated with the higher percentage of ß-1,3-glucan and chitin in thinner hyphal cell walls of 3-4 h germinating cells (188,294,322).

Lysis of the hyphal cells may have been the result of biased Zymolyase digestion of the mother cell/hyphal cell structures in combination with possible turgor forces of cytoplasmic movement during hyphal formation. Models for germination events have suggested that the cytoplasm of a C. albicans cell migrates into and forward with the extending hyphal tip and results in a highly vacuolated mother yeast cell (169). Scanning electron microscopy of germinated cells indicates that mother cell walls exhibit indentations possibly due to the vacuolation and intracellular cytoplasmic movement (59).

Minor modification of the DNA extraction procedure for C. albicans hyphae may be possible to circumvent the spheroplast lysis problem. The following two observations support that possibility. First, chromosomal DNA from Candida hyphal form cells was prepared without the removal of Zymolyase from the embedded agarose-Candida mixture and indicated no reduction of chromosomal DNA quality. This suggests that the presence of Zymolyase did not inhibit preparation of high quality chromosomal DNA, and was likely digested by proteinase K in the procedure (Figures 18 and 19). Second, DNA from yeast spheroplasts was occasionally extracted by adding lysis buffer directly to pelleted and unwashed spheroplasts, without noticeable reduction of DNA quality (some DNA samples included in Figures 9 and 14). Normally, spheroplasts were

washed twice in the sorbitol-EDTA solution before lysis and DNA extraction. These observations suggest that DNA extraction of the Zymolyase-lysed hyphal incubation mixture may be possible by omitting the spheroplast wash steps, and proceeding directly to proteinase incubation and subsequent steps.

Considerations for Candida RFLP analysis. Factors, such as cell wall susceptibility to enzymatic digestion and osmotic stability, affect DNA recovery by spheroplast extraction and are critical considerations for clinical application of C. albicans RFLP typing. In almost all Candida RFLP molecular studies published since 1987, Zymolyase digestion of cells grown in a complex medium (o/n in YPD, 37° C) has been used for spheroplast preparation under sorbitol osmotic conditions similar to this work (96,97,117,118,120,121,124,333). Though DNA extraction problems would not be expected for the predominantly yeast form C. albicans cells, the length of the overnight incubation (not specified) may permit late log or stationary phase to be reached. The reduction of spheroplast efficiency, especially by stationary phase complex media cultures, may potentially be similar to the C. albicans B311 50% drop shown in Figure 2.

This work also indicates that germinated cells, which may be present in above described 37° C cultures, may inadvertently be lysed and the DNA lost during the usual centrifugation and spheroplast wash steps. The stability of other C. albicans polymorphic forms during Zymolyase DNA extraction has not been addressed. C. albicans pseudohyphal forms were not tested for stability during these studies and none of the reports describing spheroplast DNA extraction of C. albicans switch

phenotypes (eg., white-opaque) have mentioned spheroplast stability, Zymolyase digestion efficiency, or DNA recovery (123,125).

RFLP analysis of Candida DNA, either by EtBr-stained DNA fragments or Southern blot hybridization, requires high quality DNA and sufficient sample loaded per lane to provide the banding patterns. Most studies have not specified the amount of DNA per lane when EtBr-DNA banding or probe hybridization patterns were analyzed (95-98,117,118,121,126). Only a few studies have identified amounts: 10 µg DNA per lane for HinfI EtBr patterns (120); and 2.7 µg DNA for Ca3 mid-repeat probe and 4 µg for the actin gene probe of Southern blots (49,124). Because RFLP DNA typing of C. albicans isolates has received widespread support and interest, clarification of expected DNA recovery from spheroplasts and amounts required for acceptable banding patterns would be beneficial for clinical application. If 50% DNA recovery is common for spheroplast extraction (with 95 - 100% spheroplasts), then isolates with low spheroplast efficiency or with a portion of germinated cells may present DNA recovery problems for small scale C. albicans cultures used in clinical RFLP analysis.

Ribi Cell Fractionation
and Autolysis DNA Extraction

In addition to spheroplasting, mechanical breakage by Ribi Cell fractionation and the autolysis method are cell wall breaking procedures that can be used to obtain high quality DNA from C. albicans (Figure 1 and (298)). The mechanical breakage methods circumvented consideration of culture medium, growth form, growth stage, and isolate-associated variable susceptibility, mentioned above. These two methods have been

used for preparing DNA from Neurospora hyphae (240,241) and were modified for extracting DNA from C. albicans (99). The Sorvall Ribi Cell apparatus is similar to a French Pressure Cell except that greater pressure (35,000 psi) obtained by the Ribi apparatus enables breakage of unfrozen cells. Ribi Cell fractionation was used routinely on all forms of C. albicans cells, and was especially applicable to pseudohyphal and hyphal form cultures (Figure 17 and 21).

As C. albicans cells pass through the micro-orifice of the Ribi release valve, they are subjected only briefly to shear forces and emerge with few cracks (1 - 2) in the cell wall of most cells (J. Cutler, unpublished EM observations). Shear forces, experienced by the DNA molecules, are minimized when the cell walls are broken with the Ribi fractionator. By this method, release of C. albicans intracellular contents occurs rapidly and more gently than by small glass bead (0.25 - 0.45 mm) homogenization, where samples are subjected to multiple-hit phenomena (eg., 5 min) which result in heavy cell wall fragmentation (284,334-337). Large glass beads (4 mm) are used in the autolysis method, in order to limit multiple-hits and subject the DNA to less shear force than with small beads.

For general release of C. albicans intracellular components, mechanical breakage (by Ribi Cell fractionation) alleviates the enzymatic digestion period which may pose problems in experiments designed to track transient intracellular events. C. albicans intracellular structures remain intact for $\geq$ 30 min during any enzymatic digestion. The Candida cells would likely be similar to metabolically active S. cerevisiae spheroplasts, which synthesize RNA and protein at

nearly the same rate and DNA at half the rate of similarly incubated whole-cells (338). In addition, most cell wall enzyme preparations are crude (eg., helicase, Zymolyase, and glusulase) and may introduce exogenous proteinase or other undesirable enzyme activities into the system.

C. albicans MspI Analysis

MspI Fragment Distribution

MspI restriction digests of high quality C. albicans DNA samples produced molecular weight fragments larger than expected for a 4-base cutter (see Appendix B for analysis and theoretical considerations). Some of the large molecular weight fragments appeared repetitive as suggested by EtBr staining intensity, such as the 3 - 4 kbp triplet bands and larger bands described in Figures 1 and 15. Mitochondrial DNA and rDNA sequences have been suggested to contribute almost all of the repetitive DNA components in C. albicans (127,236), but the following evaluation suggests differently.

Repetitive DNA Estimates

Using estimates reported in a review of Candida genetics (127) (actual data unpublished), the calculated amount of repetitive DNA contributed by 40 - 80 copies of the 12 kbp rDNA repeat and 30 copies of 40 kbp mtDNA per cell would approximate 2,160 kbp. The cloned mid-repeat fragments 27A and 24A (12 kbp total, 10 copies per cell) (126), Ca3 (about 9.3 kbp, 20 copies per cell) (123), and Ca7 (about 370 bp, 30 copies per cell) (98) would account for about 317 kbp total. Riggsby et al. found that 13% of the 36 - 39 mbp C. albicans genome (range of 37 -

40 fg/diploid cell) was repetitive (239,252), which indicates 4,680 - 5,070 kbp of total repetitive sequences are present. These rough calculations indicate that half the repetitive C. albicans DNA sequences are unaccounted for by mtDNA, rDNA, and cloned repeats.

Purified mitochondrial DNA from three isolates of C. albicans (Ca1, Ca105, and 9938) yielded MspI fragments which were smaller than 4 kbp (mtDNA from Ca1 shown in Figure 16, panel A, lane 2 of MspI digested DNAs, GEL program estimates (307)). The mtDNA x MspI fragments were similar in size to the triplet bands of total DNA digests (Figure 1 and 15). Therefore, the larger putative MspI repeats in total DNA of the same isolates could not be accounted for by mtDNA fragments (Figure 9, panel A, lane 2 - Ca1, lane 6 - Ca105, lane 8 - 9938). The size of rDNA in MspI digests of total C. albicans DNA was ≤ 1.5 kbp (J. Cutler, unpublished observations). These observations regarding MspI-digested mtDNA and rDNA indicate that the putative MspI repeats larger than 4 kbp in C. albicans DNA would be non-rDNA and non-mtDNA sequences, and may contribute some of the unaccounted repetitive sequences of Candida DNA.

Genomic Methylation

Several different methods are used for detecting 5-methylcytosine (5mC) bases: methylation sensitive isoschizomer restriction enzymes (eg., MspI and HpaII differentially detect 5mC in CCGG sites) (339-341); two-dimensional paper chromatography to detect ^{14}C-labelled 5mC in extracts of cells incubated with [methyl-^{14}C]S-adenosyl methionine, which donates the methyl group to 5mC (342,343); liquid or gas chromatography detection of 5mC from DNA samples (341,344-346); and, exact positions of 5mC bases may be determined by sequencing or

ligation-mediated polymerase chain reaction (PCR) [(347), and reviewed in (344)]. 5mC usually occurs in CpG dinucleotides, which are associated with regulatory sequences in some genes [reviewed in (348,349)].

C. albicans DNA analysis by gas chromatography-mass spectrometry (GC-MS) has showed low levels of genomic 5mC, but twice as much in yeast form (0.11 mol% 5mC) DNA as mycelial (0.045 mol%) DNA (346) (discussed in Appendix A). The large range of MspI fragments and prominent C. albicans banding patterns indicated that methylation changes, possibly involved with morphogenic events, may be assessed by appropriately digested EtBr-stained DNA samples.

In these studies, the isoschizomers MspI and HpaII did not indicate any EtBr-stained DNA band differences for yeast form DNA compared to mycelial form DNA (eg., Figure 17). Though not all CpG changes would occur in CCGG sites, the technique may have questionable sensitivity for low level 5mC changes, especially if the CpG modifications were associated with unique, rather than repetitive, DNA sequences. Other fungal DNAs have shown significant MspI verses HpaII DNA band differences for various developmental forms (eg., Physarum and Neurospora), but they also exhibit larger percentages of genomic 5mC (0.5 - 7%) (341,343,345,350,351). Detection of specific methylation changes in C. albicans DNA will likely require the additional sensitivity provided by specific probe hybridization techniques or ligation-mediated PCR (347).

For the following reasons, demethylation events in C. albicans DNA could not be responsible for activation of putative morphogenesis genes

during the initial events of a yeast to hyphal transition. No enzymes have been identified which will demethylate 5mC residues; therefore, DNA replication provides the only known way of establishing DNA strands without methylated bases (352,353). DNA methyltransferases use the parent strand methylation pattern to direct methyl group addition onto newly synthesized strands in a process called maintenance methylation (352,353). The absence of maintenance methylase activity results in unmethylated sequences. DNA replication in germinating C. albicans cells occurs just prior to the first septal division within the hyphae (205) indicating that any possible demethylation-associated gene activation would follow germination induction. GC-MS measurements of 5mC in 20 h hyphal cultures (346) would indicate that DNA replication without full maintenance methylase activity was occurring, and may suggest that demethylation-associated gene activity accompanies later hyphal growth.

De novo methylation, the process of adding methyl groups to previously unmethylated sequences, occurs at promoter region CpG sites and corresponds to silencing of some genes during eukaryotic development (349,354,355). De novo methylase activity does have the potential of silencing possible C. albicans yeast-phase specific genes within the time frame of germination induction events. But, methyl group addition during the transition from yeast form to hyphal form, does not fit the indicated general reduction of 5mC content in C. albicans hyphal form DNA (346).

pHpaCa7 Analysis

Plasmid Evaluation

Plasmid pHpaCa7 was generated by Cutler and Horn (99) during attempts to clone repetitive MspI fragments from C. albicans. Based upon the length of the genomic fragment to which the cloned insert probed (Figure 4), it was reasoned that the size of the insert was approximately 3.6 - 3.8 kbp. Confusion developed over the actual size of C. albicans DNA in pHpaCa7 because the insert, supposedly removed intact along with flanking pBR322 sequences in MspI digests, gave a size estimate of only 2.9 kbp of C. albicans DNA (Figure 3) (99).

Various reasons were considered to explain the discrepancy between the apparent size of the cloned insert (2.9 kbp), and the genomic fragment to which it probed (3.6 - 3.8 kbp). Deletion of an insert region may have occurred through recombination with pBR322 sequences, as has documented for A-T rich sequences cloned into pBR322 (356). The C. albicans genomic fragment may have contained methylated internal CCGG sites that became unmethylated in the prokaryotic host and, thus, susceptible to MspI digestion.

None of the postulated reasons were true, as a 2% agarose gel of pHpaCa7 x MspI and subsequent Southern blots (Figure 7) indicated that two additional Candida fragments (M and S) were present in pHpaCa7, and that the largest insert piece (L) did not have attached pBR322 flanking sequences. The additional insert pieces were inconspicuous in the initial 1% agarose gel of miniprep DNAs (Figure 3).

Further estimates from multiple gels indicated that the cloned L fragment size (3.80 ± 0.180 kbp, Table 3) was in good agreement to

genomic copy estimates in pattern ◊III isolates like C. albicans 9938 (3.87 ± 0.06 kbp, Table 4). Retrospective analysis of early size estimates for the L fragment revealed higher standard deviation in the GEL program results. Figure 3 and similar gels ranged from 200 - 500 bp for standard deviation values. A different electrophoresis unit with thinner wells was utilized for the estimates included in Table 3 and 4 and produced narrower standard deviations for individual gels by a factor of 10 (range 0.01 - 0.07 kbp, Table 3 and 4).

Genomic Arrangement of L, M, and S Fragments

Construction of the pHpaCa7 plasmid, by random cloning of C. albicans HpaII DNA fragments into pBR322, was done with the expectation that repetitive sequences would be more likely represented in resultant recombinant plasmids. Hybridization data for the C. albicans L, M, and S fragments of pHpaCa7 suggested some information concerning the genomic arrangement and relative repetitiveness of the fragments.

The M fragment does not appear to be collinear with the L fragment in C. albicans genomic DNA. Evidence against collinearity was indicated by chromosomal hybridization and EcoRI-digested DNA blots. The L fragment hybridized to chromosome 3, but M hybridized to the larger chromosomal group which probably contains the R, 1, and 2 C. albicans chromosomes (Figure 18, L probe in panel B, and Figure 19, M probe in panel B, lane 1). Also, EcoRI-digested 9938 genomic blots indicated different signal sizes for L hybridization as compared to the M fragment: the L probe hybridized to bands at 4.5, 4.0, and 1.4 kbp; and the M fragment produced signals at 12.5, 6.5, and 3.3 kbp (data not shown).

These observations of L and M hybridization patterns suggest that two separate HpaII fragments were ligated to the same pBR322 molecule during the original cloning. Theoretically, ligation of DNA molecules occurs in a bimolecular fashion, which favors concatemer formation when higher concentrations of compatible termini are present (296). The type of ligation product (eg., cyclized monomers, monomeric plasmid:one foreign DNA piece, or concatemers) depends upon the concentration and length of molecules in the reaction. The concentration of random-sized HpaII fragments in the ligation reaction was not defined by Cutler and Horn (99), so evaluation of intermolecular events remains speculative. The concentration of reacting HpaII and pBR322 molecules was probably high enough to increase concatemer formation by available cohesive termini.

The genomic arrangement of S with regard to L could not be determined from present data. The S fragment was indicated to be more repetitive than the L fragment as shown from chromosomal hybridization (Figure 19, panel D compared to Figure 18, panel B) and from MspI fragment hybridization (Figure 15). Some signal bands from MspI fragments (Figure 15) were of appropriate length (4.2 - 4.3 kbp) for an L plus S (Candida portion only) fragment. Genomic library screening by J. Cutler and R. Little Light indicated that clones homologous to the S fragment were readily identified (unpublished data), and that higher purity L-fragment probes (possibly now from the L fragment subclones pMCL-4 and pMCS-1) would be required to identify a library copy of the L fragment. An independent L fragment copy with intact flanking sequences would be needed to determine if an S homologous sequence was collinear.

Other uses for independent copies of the L fragment would include expression analysis and investigation of the four RFLP patterns exhibited by C. albicans isolates (Figure 9). If the weak Northern blot signal resulted from the pHpaCa7 L fragment containing only a portion of gene coding sequences (Figure 21), then another L copy (with additional flanking DNA) would facilitate gene analysis.

Characterization of the M and S Fragments

M Fragment Characteristics

Survey of Eukaryotic DNAs. The second largest MspI fragment (M fragment) contained in pHpaCa7 exhibited homology to DNA from C. krusei, C. guilliermondii, C. parapsilosis, S. cerevisiae, and N. crassa (Figure 14), but not Histoplasma, Blastomyces, or Sepedonium DNA (data not shown). Hybridization data from other blotted DNAs (Aspergillus, Chrysosporium, Cryptococcus, Mucor, and Coccidioides) were inconclusive for M homologous sequences, but all indicated positive hybridization with the combined M plus S fragment probes (Figure 13).

pBR322 Contribution to the M Fragment. Hybridization data concerning pBR322 portions of the M fragment do not correlate with sequence information from the HindIII ccw primer and may suggest that additional pBR322 sequences are present somewhere else in the M fragment besides the 3' end. Radiolabeled pBR322 hybridized to the M fragment (Figure 7), but sequence data indicated that a CCGG site was located in C. albicans DNA approximately 95 bases ccw from the 3' insert site in pHpaCa7 (Figure 27). The CCGG site found by sequencing suggests that

MspI digestion of pHpaCa7 should cut off 95 bp of C. albicans DNA plus the 3' pBR322 flank, and eliminate hybridization of labeled pBR322 DNA to the remainder of the M fragment.

Sequencing information (Figure 7) for the pHpaCa7 M fragment does not include approximately 1 kb of the 5' M region (Table 3). If complementary strand sequencing confirms the presence of an internal CCGG site, then other pBR322 sequences will likely be found in the 5'region of the M fragment. Two reports, concerning deletions and recombination events when A-T rich sequences were cloned into pBR322, suggest that A-T rich fragments have recombinational potential in pBR322 (356,357). Partial sequence information from M indicates some regions of A-T rich DNA: nucleotides in Figures 27 and 34 are 37% G+C; but the M region in Figure 33 is 47% G+C. Sequence data from the M fragment subclone, pBSMsp-M22, supports the potential of M sequences to recombine with pBR322 regions (Figure 27, and 33 - 35). Full sequence information for the M fragment will be necessary to resolve the pBR322 hybridization and CCGG site discrepancy.

M Fragment Sequence and Expression. Multiple open reading frames were identified for Candida DNA contained in the M fragment (Figures 27, 33, and 34). The coincidence of three rDNA regions (S. cerevisiae, S. carlsbergensis, and Mucor sequences from GenBank) with homology to ORF 188-->74 (c) (Figure 27), and M hybridization to the large Candida chromosome group (Figure 19) suggest that the M fragment may be from rDNA regions in C. albicans. Hybridization strength of the M fragment to Candida genomic digests, though, do not appear strong enough to

suggest high copy number associated with rDNA sequences (Figure 14, panel C, lane 12).

The M fragment hybridized to C. albicans 9938, Ca1, and 9938-DNAse treated total RNA samples (Figure 22 and 24, Northern blots) indicating that M fragment sequences are expressed in C. albicans. The signal strengths of yeast form and pseudohyphal form RNA were similar (Figure 24, M panel Ca1 Y and 9938 Y and Ps samples) and indicated that M sequences are expressed in both yeast forms and pseudohyphal forms.

S Fragment Hybridization

Survey of Eukaryotic DNAs. The third largest MspI fragment (S fragment) in pHpaCa7 displayed homology to DNA from all included Candida species, Torulopsis, Saccharomyces, Neurospora, human, mouse, Histoplasma, Blastomyces, and Sepedonium (Figure 14 and data not shown), suggesting broad-based conservation of S homologous sequences. Hybridization data from other blotted DNAs (Aspergillus, Chrysosporium, Cryptococcus, Mucor, and Coccidioides) were inconclusive for the presence of S homologous sequences, but all indicated positive hybridization when more DNA was probed with the mixture M plus S probe (Figure 13).

S Hybridization Characteristics. The S fragment, containing approximately 0.33 kbp of C. albicans DNA, appears more repetitive than either the L or the M fragments, because it hybridized to all the CHEF-separated chromosomal bands (Figure 19) and to many MspI fragments from genomic DNA (Figure 15). Of the 18 C. albicans isolates surveyed, the S probe produced 14 different MspI band patterns (Figure 19) suggesting

possible use in typing C. albicans isolates, if the S pattern of each isolate proves stable. The S hybridization to 6 or more C. albicans chromosomes suggests at least a middle-repetitive classification (≥ 10 copies per genome) for the S fragment. Southern blots of other fungi (Figure 14, and data not shown) indicate that homologous S sequences may be repetitive in other DNAs (eg., Neurospora, Sepedonium, Torulopsis, C. lusitaniae, C. parapsilosis, and C. guilliermondii).

Other repetitive C. albicans fragments with multi-band chromosomal hybridization are Ca3, 27A, 24A, and the telomeric sequence Ca7 (96,98,126). By comparison of cross-species hybridization, the S fragment appears distinct from these other Candida mid-repetitive sequences: Ca3 does not hybridize to S. cerevisiae (98); 27A indicated no signals from C. tropicalis and C. parapsilosis (126); 24A was weakly positive to C. tropicalis (no other details available) (126); and Ca7 gave no signals for S. cerevisiae, C. tropicalis, C. krusei, and C. glabrata (98). The S fragment hybridized to all of the above species.

S Fragment Expression. Northern blots of C. albicans RNA exhibited two specific signal sizes of 8.48 and 6.14 kbp for S homologous transcripts (Figure 22). With the indicated repetitive nature of S homologous fragments in Candida DNA, the possibility exists that the two transcripts may contain different copies of the S fragment rather than the one contained in pHpaCa7. The two large transcripts may be from separate coding regions, or the smaller RNA may possibly represent a processed version of the larger.

The signal strength for 20 µg Ca1 yeast form RNA and 20 µg 9938 pseudohyphal form RNA were similar in Northern dot blots, but comparison

of equivalent amounts of Ca1 and 9938 yeast form RNA indicated about five-fold greater expression in Ca1 than 9938 cells (Figure 24). Identification of the transcripts involved and evaluation of expression levels would be necessary to determine the significance of lower levels of homologous S transcripts in 9938 yeast forms.

S Sequence Information. Sequence information was obtained for 340 bases of C. albicans DNA and represents the approximate length for the Candida portion of S fragment (Table 3 and Figure 8). Apparently, the MspI site between the S and L fragments would be located very near the end of available sequence information.

The S fragment was 61% G+C rich, primarily because of the CAG repeat region (Figure 26). Though the CAG repeat region was not indicated to be in an open reading frame (Figure 26), many transcribed sequences on file at GenBank were homologous to that region of the S fragment (Table 5). If the CAG repeat region of the S fragment were transcribed and translated, the RGYCAG (R = A or G, Y = C or T) tandem repeat could code for amino acids in all three reading frames: serine or glycine and glutamine for RGYCAG; valine or alanine and arginine for GYCAGR; and, serine or proline and glutamic acid or glycine for YCAGRG. The first reading frame option would result in a glutamine-rich region, which may be similar to the described glutamine-rich activation domains of transcription factor Sp1, products of homeobox genes, transcribed repetitive elements in Drosophila (*opa* repeats), and an Eimeria tanella antigen gene (282,358-360). Regardless of whether or not the CAG region is transcribed in C. albicans, the apparent similarity of the CAG repetitive region to other conserved motifs probably accounts for

finding S homologous sequences in many of the eukaryotic DNAs screened in this work.

Characteristics of the L Fragment

L Hybridization to C. albicans DNA

L fragment hybridization sizes to various MspI-digested C. albicans DNAs were 3.58, 3.87, and 4.19 kbp (Table 4) which were similar to MspI triplet band sizes (Figures 1, 9, and 15) and to the larger mtDNA MspI fragments (Figure 16). These initial results suggested that the L fragment may be repetitive and possibly a mtDNA sequence. L fragment copy number, established by dot blot hybridization and chromosomal hybridization, was 1 - 2 per haploid genome (Figures 18 and 20). Nuclear origin for the L fragment was indicated by L probe hybridization to whole cell DNA extracts but not to purified mtDNA (Figure 16). Therefore, despite the implications based on size similarity, closer examination indicated that the L fragment was a unique nuclear sequence.

RFLP Patterns. Four patterns of L hybridization to MspI-digested C. albicans DNA were observed in the 50 isolates screened (Figure 9), but other RFLP sizes may be found in additional C. albicans isolates. A clustering effect in RFLP frequency was observed in that approximately half of the isolates exhibited the doublet pattern ◊I as compared to patterns ◊II, ◊III, and ◊IV. This trend of C. albicans isolates to exhibit predominantly similar RFLP patterns or other biotyped characteristics has been observed by others (117,119).

Evaluation of pattern sizes revealed a noticeable size correlation between pattern ◊I doublet and the singlet sizes of patterns ◊II and

◊III (Table 4), as if pattern ◊I was a combination of other two patterns. Two L fragment sizes in the doublet pattern may suggest that chromosomal homologs contain a particular sized copy, whereas the singlet patterns may have the same L copies on both homologs. Because C. albicans isolates are asexual, investigation of proposed homolog differences may be done by protoplast fusion followed by segregant hybridization analysis (321,361). Disruption mutagenesis would be an alternative approach, if available C. albicans auxotrophs displayed the doublet L pattern (221). Independent copies of the L fragment from other RFLP patterns (other C. albicans 9938 pattern ◊III) would be necessary to determine sequence level changes accounting for the 200 - 300 bp size differences between each band (Table 4). The pattern ◊IV singlet was not observed in a doublet pattern for isolates screened, but may be found in a doublet RFLP pattern by testing additional isolates.

Consideration of methylation differences to explain the 4 RFLP patterns can only account for bands larger than 3.87 kbp band in 9938 DNA. The L fragment copy from 9938 probably does not contain an internal CCGG site, otherwise it would be digested by MspI after replication in a prokaryotic host. Both terminal CCGG sites of the L fragment are unmethylated in genomic 9938 DNA, because MspI- and HpaII-digested DNA produces the same sized hybridization signal (Figure 17). Methylation of a terminal CCGG site(s) of the L fragment could account for the appearance or presence of the larger 4.19 kbp signal in some C. albicans isolates. But, the smaller 3.58 kbp band size can not be explained by a methylation difference; the simplest proposed difference

may be that the small L copies are missing about 300 bp compared to L fragment copies in 9938 DNA.

L Fragment Expression and Sequence

Northern blot analysis from formaldehyde agarose gels (Figure 21, panel B), total and mRNA-enriched sample dot blots (Figure 23, panel A), and DNAse-treated total RNA samples (Figure 24) provide evidence that L fragment sequences are expressed in yeast, pseudohyhal, and mycelial forms. Transcript mapping will be necessary to determine if all coding regions are contained in the L fragment. C. albicans genes appear to have unusual exon and intron break capacity, even though the splicing signals for RNA transcripts are normal (236). Recently the calmodulin gene was found to have a 222 bp intron immediately after the Met start codon (362). If an L fragment transcript(s) is translated, it may provide a C. albicans-specific antigen or a target for therapeutic advantage. Though unique DNA sequence does not guarantee a uniquely functioning protein, the possibility exists for a 3.87 kbp region to provide a translated product.

Partial sequence information obtained for the L fragment subclones pMCL-4 and pMCS-1 indicated A-T rich DNA (35% G+C) similar to C. albicans genomic values, and only 6 of 17 ORFs longer than 20 amino acids (Figures 29 - 32). FastA searching of GenBank files indicated limited regions of homology considering the number of possible ORF regions: the 80 bp region homologous to chicken prostaglandin synthase did not overlap the one indicated ORF in pMCL-4 RP sequence; and, one ORF of pMCS-1 RP (28-->1 c) was responsible for 4 short regions (all < 51 bp) from GenBank sequences. The FastA search included all

databases containing nearly 58,000 sequences on file at GenBank (September 1991) including bacterial, plant, organelle, invertebrate, mammalian, structural RNA, and viral sequences.

Consideration of Species-Specific Traits

Throughout this work, L fragment hybridization was positive only to isolates of C. albicans and C. stellatoidea. Twenty other eukaryotic DNAs from diverse genera were all negative, including six other Candida species and other medically important fungi (Figures 10 and 11). As a result of these screenings, the 3.87 kbp L fragment from C. albicans 9938 appears to have restricted hybridization and represents the most extensively tested species-specific fragment of C. albicans.

Comparison to other Species-Specific Fragments. The term species-specific, with regard to Candida fragments, is used to indicate two different meanings. The first sense, or type 1, conveys "species-distinguishing" or "strain-distinguishing" capacity, signifying that fragments hybridize to more than one Candida species and are capable of distinguishing between species or isolates of C. albicans on the basis of unique RFLPs. The second usage, or type 2, denotes restricted hybridization of that fragment to only C. albicans DNA and is, thus, "species restrictive". The L fragment, described here, falls into the species restrictive type of species-specific sequences. Table 6 lists fragments which are currently identified as species-specific and describes their particular characteristics for comparison.

Table 6. C. albicans species-specific fragments.

Sequence	Type[a]	Reference	Organisms Screened[b]
Ca3 [mid-repeat]	1	(123,365) (98,124)	+ *C. albicans* + *C. tropicalis* + *C. stellatoidea*
Ca5 [rDNA]	1	(97,98)	+ *C. albicans*, + *C. stellatoidea* + *C. tropicalis*,+*C.guilliermondii* + *C. parapsilosis*, +*C. glabrata*[c] + *S. cerevisiae*
Ca7[d] [telomeric]	2	(98,125) (123)	+ *C. albicans*, + *C. stellatoidea* - *C. tropicalis*, - *C. krusei* - *C. glabrata*[c], - *S. cerevisia* - *Tetrahymena*
ACT1 [actin gene]	1	(95)	+ *C. albicans*, +*C. stellatoidea* + *C. guilliermondii*, + *C. krusei* + *C. tropicalis*, +*C.parapsilosis* + *C. kefyr*, +*T. glabrata*[c]
CAP [aspartyl proteinase]	2	(49)	+ *C. albicans*, - *C. tropicalis* - *C. parapsilosis*, - *C. krusei* - *C. guilliermondii*, -*C.shehatae* - *C. lipolytica*, - *C. utilis* - *S. cerevisiae*
27A	1 & 2	(126,363)	+ *C. albicans*, - *C. tropicalis* - *C. parapsilosis*
L	2	(99) This work	+ *C. albicans*, + *C. stellatoidea* - *C. tropicalis*, - *C. lusitaniae* - *C. guilleirmondii*, -*C. krusei* - *C. parapsilosis*, - *C. kefyr* - *S. cerevisiae*, - *T. glabrata*[c] - *N. crassa*, - *Histoplasma* - *Blastomyces*, - *Sepedonium* - *Chrysosporium*, - *Cryptococcus* - *Coccidioides*, - *Mucor* - *Aspergillus*, - *Trichomonas* - *Gaeumanomyces* - human DNA, - mouse DNA

[a] Type refers to either: 1 = strain or species-distinguishing
2 = species restrictive

[b] Organisms screened: + indicates positive cross-hybridization
- indicates negative cross-hybridization

[c] C. glabrata and T. glabrata, controversial designation (8)

[d] Ca7 was not described as species-specific by its investigators, but does qualify by negative hybridization pattern to other organisms.

Ca5, *ACT1*, 27A and especially Ca3 sequences have had substantial impact upon DNA typing of Candida species and strains. Epidemiologists frequently use DNA typing in conjunction with other biotyping methods to assess questions such as the personal carriage of C. albicans strains and tracking nosocomial outbreaks (96,97,117-119,121,123-126,363-365).

Type 2 species-specific fragments 27A, *CAP*, and Ca7 have provided valuable information concerning C. albicans genetics and biology. 27A was the first cloned mid-repetitive element which had high mutation rates like transposable elements (126,363). The telomeric sequence Ca7 has provided insight into features of the C. albicans variable telomeric organization and may provide a tool for investigating the variable karyotypes of C. albicans and C. stellatoidea (98). The cloned *CAP* gene may aid definition of the specific role of aspartyl proteinase in the pathogenesis of Candida species (49).

DNA Diagnostic Probe. An undeveloped potential for type 2 species-specific fragments involves use as DNA diagnostic probes for rapid detection of C. albicans in clinical samples. Diagnosis of disseminated candidiasis is problematic at various levels (described in the introduction), including the time required for positive blood culture results. Development of a DNA tool for quicker positive identification may prove useful, especially in patients at-risk for opportunistic infection. A C. albicans DNA probe would not necessarily distinguish between a contaminated specimen and disease-associated presence, but identification through DNA hybridization would not depend upon outgrowth of the cells in the specimen. The culturability of C. albicans from

post-antifungal treated individuals may be especially low as seen in patients with chronic disseminated candidiasis (366-368).

Hybridization specificity to the target organism or the capacity to produce fingerprint RFLP patterns are primary qualifications for the consideration of any candidate DNA probe sequence (92,93,369,370). Repetitive DNA sequences are favored for specific hybridization use, but amplification of target sequences (eg., PCR) or signals from probe:target complexes allow single copy sequences to be useful as diagnostic probes (371,372). Type 2 species-specific DNA sequences would facilitate a positive/negative signal assay and eliminate electrophoresis required in C. albicans RFLP patterns from type 1 species-specific sequences.

27A, *CAP*, Ca7, and the L fragment have shown species-specific hybridization against the various DNAs noted in Table 6. Soll et al., and Sadhu et al, have not used the term C. albicans "species-specific" for telomeric Ca7, but hybridization of the fragment was restricted to C. albicans and C. stellatoidea. The five other DNAs which were negative for Ca7 hybridization (98,125) represent a longer survey list (only two other Candida species) than reported for 27A, which was specifically called "species-specific" (126,363). The reported range of organisms probed with Ca7, 27A, and CAP is more limited than L fragment described in this work.

Clinical samples, such as lysis-centrifuged blood cultures, contain a multitude of human DNA, cell debris, and possible other microorganisms besides C. albicans (eg., bacterial pathogens or contaminants, other fungi, and viruses). It was not the intent of this work to eliminate

all possible cross-hybridizing DNAs, but to investigate the nature of L fragment hybridization to other probable fungi and to eukaryotic DNA that might be present in clinical samples. Though the viral and prokaryotic DNAs would require testing before development of diagnostic application, strong indications from these studies support C. albicans species-specific status for the L fragment among eukaryotic DNAs.

Conclusions

1) Ribi Cell fractionation, autolysis, and spheroplast DNA extraction methods (298) were equivalent at providing high quality DNA from C. albicans. The mechanical breakage methods eliminated consideration of culture medium, growth form, growth stage, and isolate-associated differences in cell wall susceptibility to Zymolyase digestion. Multiple, small-scale samples were more easily processed by spheroplast methods, whereas large volume extractions were routinely done by Ribi Cell fractionation. Modification of the Zymolyase digestion and spheroplast extraction method would be necessary to extract DNA from C. albicans hyphal forms.

2) MspI and HpaII restriction digests of high quality C. albicans DNA samples produced molecular weight fragments larger than expected for 4-base cutters. The large molecular weight MspI/HpaII fragments are probably A-T rich and devoid of unmethylated CCGG sites. EtBr-stained intensity of some bands suggests that repetitive sequences larger than 4 kbp may be present which are not rDNA or mtDNA sequences.

3) The extended hybridization survey, included in this work, supports the C. albicans and C. stellatoidea species-specific status for the L

fragment from pHpaCa7 (99). The fragment shows potential use as a diagnostic probe because it did not hybridize to other Candida species, other medically important fungi, or mammalian DNAs. The L fragment, at 1 - 2 copies per genome, exhibited A-T rich nucleotide sequence with ORF regions, indicated no methylation changes at fragment-terminal CCGG sites during yeast to mycelial transition, and displayed positive hybridization to yeast and mycelial form RNA extracts from C. albicans. 4) Two additional C. albicans DNA fragments, M and S which were unexpectedly present in pHpaCa7, caused confusion regarding the size of the L fragment and the initial restriction map (99). The M and S fragments were partially characterized by sequencing and both indicated positive hybridization to Northern blots of C. albicans RNA. The S fragment cross-hybridized with many other eukaryotic DNAs and may be useful in C. albicans strain differentiation.

APPENDICES

APPENDIX A

GROWTH OF *C. albicans* 9938 IN DEMETHYLATING COMPOUNDS

GROWTH OF C. albicans IN DEMETHYLATING COMPOUNDS

Eukaryotic Methylation and Cytosine Analogs

Two compounds, 5'-azacytidine (5AC) and 5'-aza-2'-deoxycytidine (5AdC), can be incorporated into replicating DNA as nucleoside analogs that inhibit eukaryotic methylation of cytosine residues into 5-methycytosine (5mC) (373-376). The extent of demethylation usually exceeds that of incorporation alone and may be due to inhibition of methyltransferase enzyme activity by direct binding of the analogs (314,377). DNA methyltransferases perform maintenance methylation, that occurs in a semi-conservative fashion upon newly synthesized DNA strands in replication, and *de novo* methylation that establishes new methylation patterns on previously unmethylated sequences (374,378-380).

5AC and 5AdC have been used to investigate demethylation of CpG sites upstream of developmentally regulated genes (314,374). Vertebrate CpG dinucleotides occur at 1/5 of the expected random frequency in DNA, and sometimes occur in clusters, or "CpG islands", in promoter region of genes [reviewed in (348,349)]. The association of actively transcribed areas of vertebrate genomes and unmethylated CpG islands has been established; but, methylation as a means of gene regulation in all eukaryotes has not been demonstrated (344,353,374,381-383). Inhibition may occur through 5mC-binding proteins interfering with transcription factor binding events (384).

Fungi appear to be a heterogeneous group regarding the incidence and amount of genomic 5mC content: Aspergillus nidulans and S. cerevisiae (<0.01 mol% 5mC) have undetectable levels of genomic 5mC; Phymatotrichum omnivorum (plant pathogen, 0.78 - 2.27% of total cytosine); Phycomyces blakesleeanus (0.5 - 2.9% of total cytosine); Physarum polycephalum (slime mold, 4 - 7% of total cytosine); Mucor rouxii (detected by isoschizomers MspI and HpaII digestion); and N. crassa (0.24 - 0.40 mol%) (342-344,350,385-390). (5mC content values are expressed either as moles percent of total bases (mol%) or as the percentage of cytosines that are methylated.) All of the fungi with measurable levels of 5mC listed above, have levels which differ according to developmental stage. Limited information on 5mC content in C. albicans, described below, suggests that measurable 5mC levels are present and may change during morphogenesis.

Methylation and C. albicans

A literature report by Pancaldi et al., indicated that 5AC treatment of C. albicans promotes germination (391). Cells exposed to 10 mM 5AC for 24 h (27^0 C in Sabouraud dextrose broth), starved o/n in citrate buffer (27^0 C), and placed in fresh medium with 20% calf serum (37^0 C) germinated faster (85% at 1 h, nearly 100% at 2 h) and displayed more uniform germ tube lengths than untreated controls (13% germinated at 1 h, 36% at 2 h, and 90% at 5h). 5AC grown yeast cells did not differ microscopically from untreated yeast cells. Concentrations of 0.1 mM and 1 mM 5AC did not induce the germination rate effect seen at 10 mM. The germination studies were done in triplicate on three

occasions, but only one C. albicans isolate was included (391). No reason was given for the high concentrations of 5AC (0.1 - 10 mM) which contrast markedly with 1 - 100 μM 5AC used in mammalian cell treatments (392) or 6 - 48 μM 5AC exposure for Neurospora (393).

Induction of C. albicans hyphae can be influenced by various factors including the metabolic history of the culture (eg., growth phase and carbohydrate source) [reviewed in (8)]. Stationary phase cells germinate more readily than log-phase C. albicans cells which often require incubation under starvation conditions in order to germinate (8). No observations regarding growth rates of C. albicans in 5AC, as compared to untreated controls, were given by Pancaldi et al., but they did state that starved, 5AC-grown cells had yeast cell growth rates similar to untreated controls when cells were inoculated into fresh Sabouraud dextrose broth without serum.

In their experiments the o/n starvation in 27° C citrate buffer would presumably equalize any differences in growth phase for the 5AC treated and untreated cultures, but an important control to support that presumption was missing. A log-phase versus stationary phase control for both 5AC treated and untreated cells should have been run to determine if citrate buffer starvation equalized the germination rates. The capacity of 5AC treated and untreated cells to produce similar yeast form growth rates would not necessarily establish that all variables during germination induction were the same except 5AC treatment.

Though their work provocatively suggests that demethylation speeds up germination, the possibility exists that 5AC treated cells may have been in a growth phase (eg., stationary phase) more conducive to

germination than untreated cells rather than the assumed demethylation effects of 5AC. Suggestions by the investigators that demethylation events promote rapid germination and environmental factors, that induce dimorphic transition in C. albicans, may trigger DNA demethylation remain speculative because of considerations and controls not addressed by their experiments.

Despite the above experimental limitations, another group of investigators reported that yeast form C. albicans contains roughly two-fold higher 5-methylcytosine than mycelial form DNA as determined by gas chromatography - mass spectrophotometry (GC-MS) (346). Russell et al. used three isolates of C. albicans that were grown in a defined medium with either 6.25 mM ammonium nitrate for yeast form growth or 100 mM ammonium nitrate and 47.5 mM arginine to induce mycelial forms. DNA was extracted from 20 h low or high nitrate cultures for analysis of 5mC content.

Under the high nitrate conditions, the isolates exhibited various percentages of mycelial growth: C. albicans 9938 produced only 44% mycelial forms (excluding pseudohyphae) with 0.053 mol% 5mC content compared to 0.11 mol% in yeast cultures; isolate Sh8 produced 73% mycelial forms at 0.045 mol% 5mC compared to 0.11 mol% in yeast cultures; and isolate B311 produced 81% mycelia at 0.047 mol% 5mC whereas yeast cultures were 0.097 mol% 5mC. Because the evaluated mycelial cultures were at least 19% yeast and/or pseudohyphae, the investigators noted that the actual mol% of 5mC in C. albicans mycelial cultures may be less than indicated (346). Predominantly pseudohyphal cultures were not analyzed to provide a comparison of 5mC content in

pseudohyphal versus yeast or mycelial form cultures. The polymorphic nature of C. albicans presents a significant limitation to obtaining 100% mycelial or pseudohyphal cultures as lateral blastospores may be produced by both forms (8).

Another practical limitation for 5mC analysis exists in the requirement of sufficient cell division or DNA replication to establish differences in methylation. No demethylase activities have been identified, indicating that sequences which are methylated must go through two rounds of DNA replication without maintenance methylation in order to establish a demethylated DNA region (314,344,373,374,394). For C. albicans, sufficient culture development for true mycelia without formation of lateral blastospores may not occur in the germination method used by Russell et al., but may be possible in 18 - 27 h Lee medium mycelial cultures (1) or in GlcNAc induced C. albicans (24,395).

The 0.11 mol% for C. albicans yeast found by Russell et al., represents a 10-fold greater amount of 5mC content than the similar sized Saccharomyces genome and suggests the potential for significantly methylated sequences. The difference in 5mC content between yeast cultures and mycelial cultures and the above 5AC studies suggest that demethylation may be involved with morphogenesis changes in C. albicans, but cause and effect relationships have not been established.

Culturing C. albicans 9938
in 5AC and 5AdC

The number of large MspI and HpaII fragments from genomic C. albicans DNA suggests these fragments are A-T rich and either have no CCGG sites or have protected sites; the latter may indicate the presence

of 5mC. To investigate if 5AC or 5AdC treatment would modify the appearance of high molecular weight MspI and HpaII EtBr-bright bands, yeast form cells of isolate 9938 were grown in the presence of each compound. Flasks containing fresh 50 ml mLee were inoculated with 1.16 x 10^5 cells/ml from an o/n 9938 mLee culture (23^0 C). Filter sterilized 5AC or 5AdC was added to give 6 µM and 24 µM concentrations (393) and foil wrapped flasks were incubated at RT for growth of yeast form cells (160 rpm). At 21 h, media containing 24µM of 5AC and 5AdC cells were spiked with an additional 24 µM of each compound. (Cells cultured in 5AC and 5AdC were not examined for germination efficiency.)

Culture densities were determined up to 48 h, then cells were harvested and DNA extracted by the spheroplast method. Growth curve plots of control versus 5AC or 5AdC cultures are in Figure 36. Error bars represent the standard deviation of two counts per flask at each sampling time. In general, 5AC and 5AdC treated C. albicans 9938 cells grew faster than the untreated control culture (Fig. 36). Repetition of these experiments with other C. albicans isolates and replicate flasks would be necessary to establish a growth rate difference.

DNA Analysis

DNA samples were digested with MspI or HpaII and electrophoresed in 1% TAE agarose (panel A, Figure 37). The HpaII digest (lane 3) of 9938 control DNA revealed a 5.37 kbp band (arrow, GEL program estimate, SD of gel = ±0.04 kbp) that was not seen in the MspI lane (lane 2) of the same sample. The band was the first MspI versus HpaII methylation difference observed for the 9938 strain (DNA extract March 1990) though J. Cutler and later, H. Horn, tested 9938 DNA for such differences (approx. 1984 -

1987). C. albicans 9938 was obtained as a clinical isolate in 1970 (J. Cutler) and has since been in laboratory subculture. Though the 9938 cells cultured in 5AC and 5AdC also showed the 5.37 kbp HpaII band (lanes 7, 9, 11, and 13), the control DNA indicates that the 5.37 kbp band methylation change was unrelated to culturing with 5AC or 5AdC.

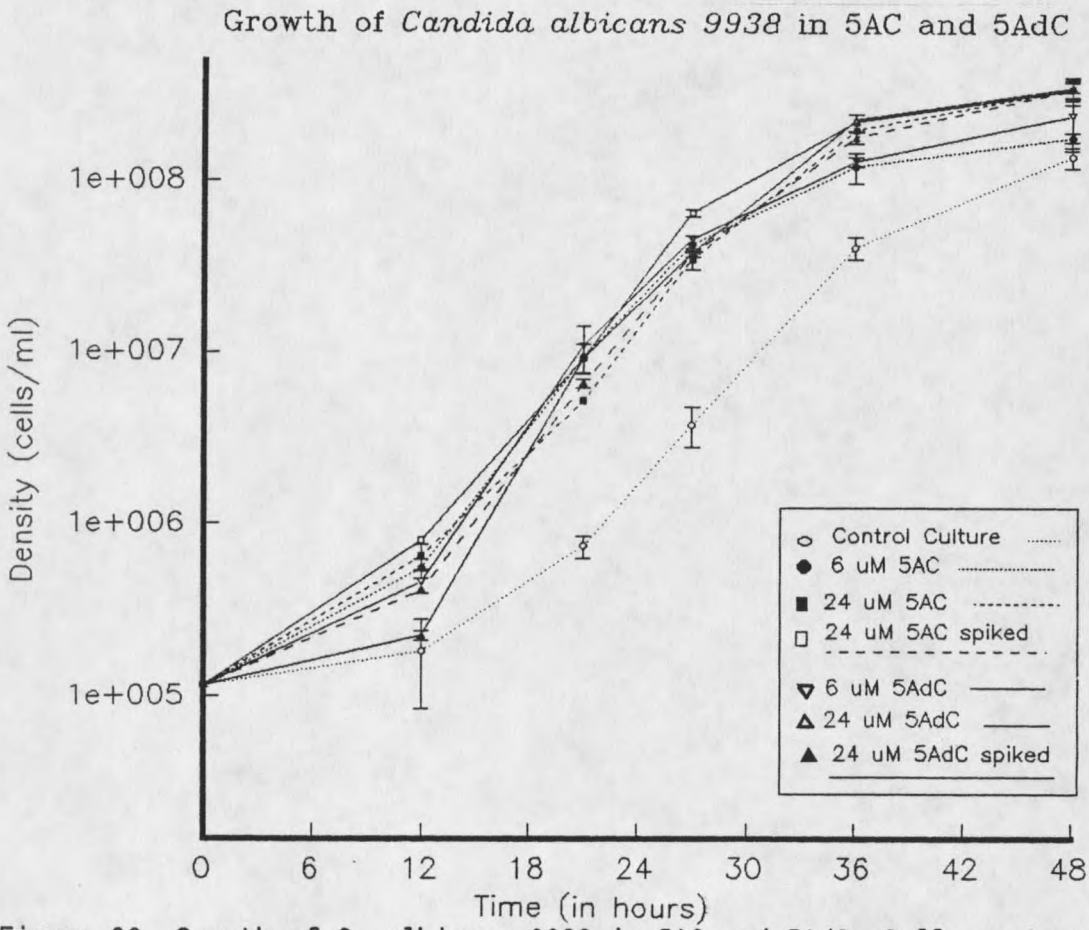


Figure 36. Growth of C. albicans 9938 in 5AC and 5AdC. Cell counts of mLee 9938 cultures, treated with 0, 6, or 24 µM 5AC or 5AdC (RT) were taken during 48 h growth before DNA extraction of the cells. The abscissa labels denote 1 x 10^5 to 1 x 10^8 cells/ml counts.

The EtBr stained gel exhibited overall faster migration for HpaII bright band fragments (lanes 7, 9, 11, and 13) of 24 µM 5AC and 5AdC samples compared to MspI digests (lanes 6, 8, 10, and 12). The shift can be seen best for the 4.72 kbp MspI bright band (lane 6 arrow) which

GEL program estimates of lane 6 and 7 indicate a 100 bp migration shift (same gel, SD = ± 0.04 kbp). DNA from the 6 µM 5AdC culture was lost during processing, and on this gel, the 6 µM 5AC loading in lanes 4 and 5 was too light to see band patterns. Untreated control DNA indicated a less pronounced migration shift for HpaII bands (lane 3 compared to lane 2, MspI). The same shifts were observed in two additional gels with independent digests of all the DNA samples (data not shown). The reason for the observed overall shift was not investigated.

The gel in Figure 37 was blotted and probed with the L fragment (Figure 37, panel B). The L probe hybridized with one MspI fragment in control 9938 DNA (lane 2) and two bands with HpaII-digested control DNA (lane 3, arrows) indicating that one terminal CCGG site of the L fragment contained a HpaII resistant $C^{m}CGG$ site. This was the only occasion (1 of 5 different 9938 DNA extracts) when the L probe pattern of 9938 was different for MspI versus HpaII digests, again indicating methylation differences for this culture (eg., Figure 17 exhibits one band for both enzymes.) The larger HpaII band corresponds to the upper band size for pattern ◊II (Table 4) suggesting that methylation may influence the L fragment RFLP patterns observed among C. albicans and C. stellatoidea isolates. L probe hybridization to the 5AC and 5AdC DNA samples digested with MspI (lanes 4, 6, 8, 10, and 12) or HpaII (lanes 5, 7, 9, 11, and 13) show the same number bands (with the above described migration shift) as control DNA lanes cut with each enzyme.

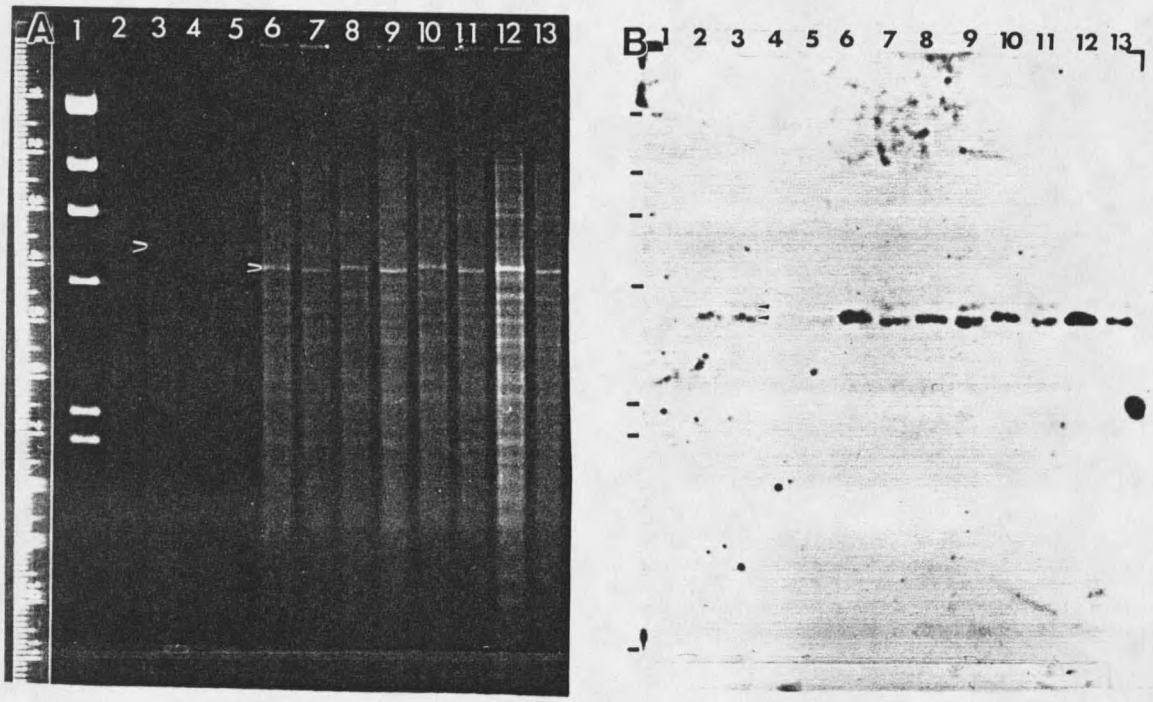


Figure 37. Gel and Southern blot of 5AC and 5AdC C. albicans DNA samples. Panel A: EtBr-stained 1% TAE agarose gel containing untreated, 5AC, or 5AdC cultured 9938 DNAs digested with MspI (even lanes) or HpaII (odd lanes). Lane 1 - Lambda HindIII marker; lane 2 (M) and 3 (H) - untreated C. albicans DNA; lane 4 (M) and 5 (H) - 6 µM 5AC: lane 6 (M) and 7 (H) - 24 µM 5AC; lane 8 (M) and 9 (H) - 24 µM spiked 5AC; lane 10 (M) and 11 (H) - 24 µM 5AdC; and lane 12 (M) and 13 (H) - 24 µM spiked 5AdC. Band arrows are described in the text. Panel B: Blot was probed with labeled L fragment. 9938 displayed two HpaII bands and one MspI band, which denotes a methylation difference for the L fragment not seen in earlier cultures of 9938 mentioned in the text.

These results suggest that the HpaII resistant $C^{m}CGG$ site remained methylated despite culturing in the presence of 5AC and 5AdC. Similar failure of 5AC or 5AdC to promote complete genomic demethylation has been observed in 5AC subcultured mouse cell lines which maintain a low level of persistently methylated cytosine residues (392). The M and S fragments were not used to probe the 5AC or 5AdC DNA samples.

These two observed differences in methylation (5.37 kbp HpaII band and the L HpaII doublet pattern) indicate circumstantial support for the measurable levels of yeast 5mC found by Russell et al., but analysis of the restriction patterns (Figure 37) do not provide 5AC- or 5AdC-associated differences for MspI verses HpaII digests to confirm that analogs were incorporated into C. albicans DNA.

APPENDIX B

MOLECULAR WEIGHT DISTRIBUTION OF MspI FRAGMENTS

MOLECULAR WEIGHT DISTRIBUTION OF MspI FRAGMENTS

The expected frequency of a restriction endonuclease with a 4 base recognition sequence would be 1 site per 256 nucleotides ($1/4^4$) assuming random base sequence. For MspI (CCGG) sites in the 32.8% G+C genome of C. albicans (238), the expected frequency would be 1/1,384 bases (the probability of encountering a G or C equals 0.164, 0.164 = 1/6.10, and 6.10^4 = 1,384) (396). MspI produces many Candida fragments larger than expected (see Figure 1, 15, and 38). Two methods were used to assess the relative proportion of high molecular weight MspI fragments as separated by gel electrophoresis. Gel photographic negatives (Polaroid, Type 55) from 1 and 1.5% agarose were analyzed by the MCID image analyzer to compare fragments from two four-base cutters, MspI and MboI (G↓ATC with expected frequency of 1/329 bases). Radiolabeled genomic C. albicans 9938 DNA was digested with EcoRI or MspI, electrophoresed in 0.8 and 1.4% agarose, and fragment distributions obtained by Cerenkov counting lane slices.

MCID Negative Scan

The MCID system was calibrated to standard optical density (OD) units and using a gel scan subroutine, a ladder sampling window was defined for lane dimensions observed on the monitor screen (eg. 480 pixels high, 10 pixels wide, and a 3 pixel increment step height). For each sample lane, the program computed an average OD value for each step

(from the 30 pixels in the 10 x 3 pixel step size) and recorded 140 - 160 steps per lane. The 3 pixel step height provided adequate resolution of individual genomic bright bands as shown in Figure 38 (eg., MboI bands near 3 kbp long in lanes 8,9, and 10 to the dotted line traces between steps 35 - 40).

Negatives scanned for MspI and MboI distributions included Figure 15 (Results section, 1% TAE agarose gel) and a 1.5% TAE agarose gel shown here in Figure 38. An MCID Asci II file of Figure 38 OD values was imported into Sigmaplot and graphed by expressing the OD step values as a percent of the total OD units per lane (to minimize variations in loading). The individual lane traces are shown in Figure 38 with solid lines representing MspI digested DNA (lanes 3, 4, and 5) or dotted lines for MboI cuts (lanes 8, 9, and 10). Lanes 2 and 7 were not included in the graph because the DNA appeared sheared in comparison to other 9938 x MspI DNA samples (eg. Figure 1 and 15). The Lambda phage DNA x HindIII standard lane was also scanned, the plot used to mark the standard positions (x's in the top marker box), and then the Lambda trace was removed for graph clarity.

The profile of DNA fragments for the three isolates were similar in Figure 38, especially for CaOs and Ca105 (labeled with circles). The Ca1 traces (labeled by arrows) exhibited shifts toward smaller fragments which may represent some shearing of the sample. MboI digested the Candida genome more frequently than MspI and produced the highest percent OD values in sizes smaller than 2 kbp. The MspI digestion profile indicates the opposite, that fragments larger than 2 kbp produced the highest percent OD values for individual steps (Fig. 38).

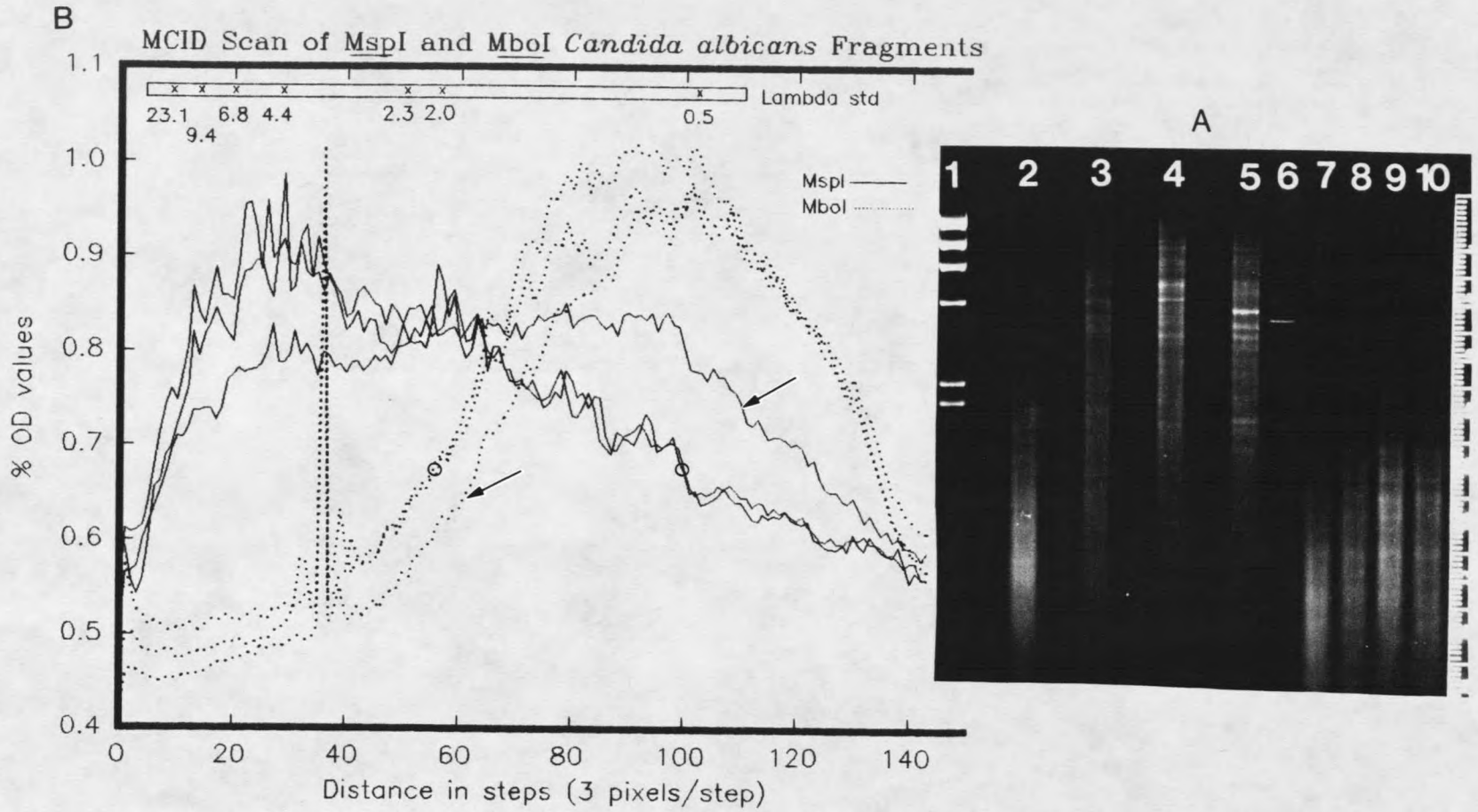


Figure 38. Gel and MCID negative scan of C. albicans DNA fragments. Panel A. 1.5% TAE gel with MspI digests (lanes 2-5) and MboI digests (lanes 7-10). Lane 1 - Lambda HindIII marker; Lanes 2,7 - 9938; Lanes 3, 8 - Ca1; Lane 4, 9 - CaOs; Lanes 5, 10 - Ca105; and Lane 6 - L fragment of pHpaCa7. Panel B. OD values were graphed to indicate the relative percent of total OD for MspI (solid lines, lanes 3-5) and MboI digests (dotted lines, lanes 8-10) for each step (3 pixel height). DNA marker (labeled in kbp) positions relative to step number are indicated in the horizontal box. Arrows label Ca1 traces and circles mark CaOs and Ca105.

The OD step values from the negative of Figure 15 were expressed as a percent of total OD per lane (like above) and the mean accumulated percent per step for 16 Candida isolates was graphed as a distribution profile for MspI fragments (Figure 39). Lambda HindIII marker scan was graphed as a dotted line. Lanes 5 and 20 were omitted from the scan because of apparent degradation. The standard deviation (SD) in Figure 39 was for individual step percent OD's and not the standard deviation of accumulated percent values. An error bar can only be seen for step 104 (SD = ±1%) because all other SD's were less than 0.5% The data indicates about 48% of the total OD per lane was above 2 kbp and approximately 23% of the total was 4.4 kbp or larger.

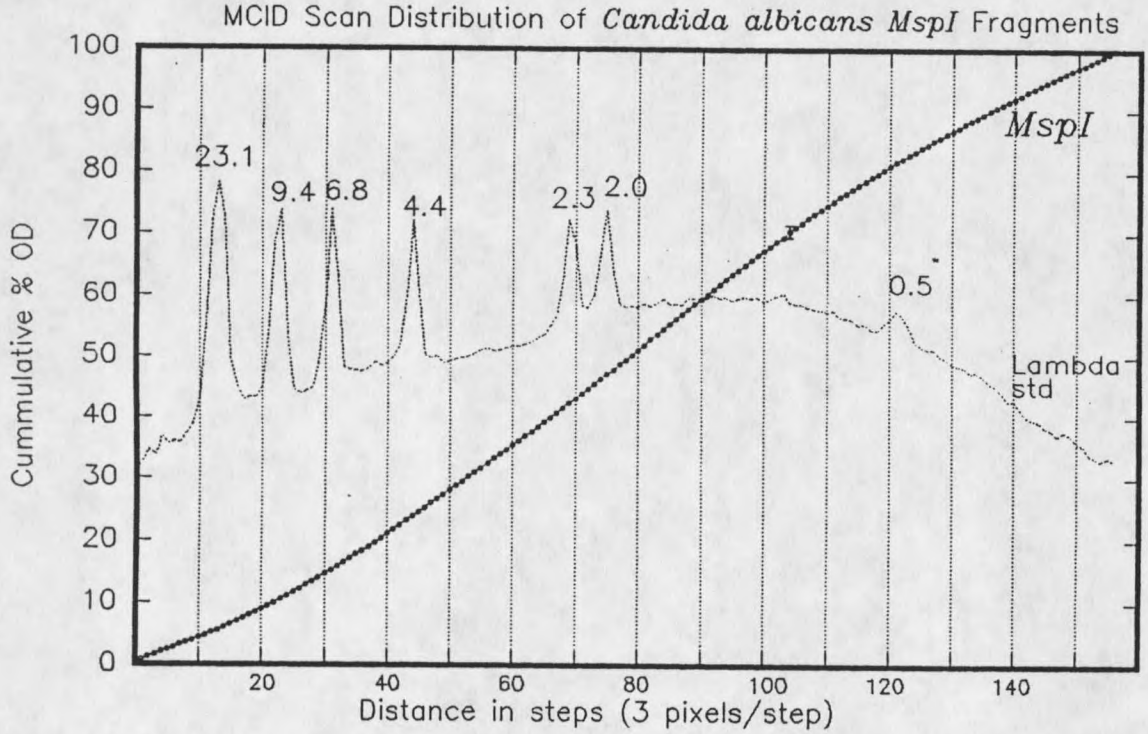


Figure 39. MCID mean distribution of C. albicans MspI fragments. Figure 15 gel negative was scanned by MCID calibrated to standard OD units with a gel window length of 156 steps per lane (3 pixel height, 10 pixels wide). Step OD values were expressed as a percent of total OD per lane, the mean (16 lanes) step values were graphed in cumulative manner with SD values from individual steps (error bar only seen for largest SD value [1%] at step 104). About 23% of the total were fragments ≥ 4.4kbp.

Radiolabeled DNA Distribution

C. albicans 9938 nucleic acids were labeled with ^{32}P-H_3PO_4 in vivo using the low phosphorus Vogel's Minimal Medium-N (VMM-N) of Metzenberg, et al. (241). The 1 mM phosphate medium proved sufficient for N. crassa growth without overloading the cell with reserve polyphosphates. Per liter VMM-N contains 125g $Na_3Citrate \cdot 2H_2O$, 6.94g KH_2PO_4 anhydrous, 100g NH_4NO_3 anhydrous, 10g $MgSO_4 \cdot 7H_2O$, 5g $CaCl_2 \cdot 2H_2O$, and 5 ml Trace Element Solution (TES). The TES contained (per 100 ml) 5g citric acid$\cdot H_2O$, 5g $ZnSO_4 \cdot 7H_2O$, 1g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.25g $CuSO_4 \cdot 5H_2O$, 0.05g $MnSO_4 \cdot H_2O$, 0.05g H_3BO_4 anhydrous, and 0.05g $NaMoO_4 \cdot 2H_2O$. The VMM-N and TES stock solutions were stored at RT with 1 - 2 ml chloroform (per 100 ml). Diluted VMM-N was autoclaved and cooled, brought to 2% glucose or sucrose (sterilized separately), and 0.04 mg/l biotin.

C. albicans 9938 cells were grown o/n in GYEP, washed twice in sterile d-H_2O, and inoculated at 8.7 x 10^4 cells/ml in 100ml VMM-N plus glucose, biotin, and 0.5mCi ^{32}P-H_3PO_4 (NEN catalogue NEX-054, 2.0 mCi/ml in 0.02 N HCl). Cultures were rotated (160 rpm) at 37°C for 15 h, harvested in log phase (2.67 x 10^7 cells/ml), and DNA extracted by the spheroplast method. The final DNA pellet was suspended in 400 µl TE buffer and a 1 µl Cerenkov count of ^{32}P emissions was 124,000 cpm/min (approximately 8.27 x 10^5 cpm/µg DNA). This specific activity was at least one log lower than a similar labeling experiment by J. Cutler using a higher specific activity ^{32}P-H_3PO_4 no longer available from ICN.

DNA samples (0.5 and 1.0µg) were digested with MspI or EcoRI, electrophoresed in 0.8% or 1.4% TAE agarose gels, lanes were cut into approximately 70 x 2mm slices (The Mickle Gel-Slicer, Brinkmann Instruments, Westbury, N.Y.), and placed into scintillation vials for Cerenkov counting. Slice counts were expressed as a percentage of total counts per lane and the mean from four lanes of MspI or EcoRI digests (two lanes each of 0.5 and 1.0 µg DNA with each enzyme) was determined.

The means were graphed as accumulated percent of total counts (with SD of individual vial counts) compared to distance migrated (Figure 40). Lambda HindIII and ϕX174 HaeIII DNA marker bands were measured from stained lanes of each gel and positions were marked on the top (1.4% agarose) and bottom (0.8% agarose) horizontal bar, seen in Figure 40.

Little difference was observed for the overall distribution of EcoRI (boxes) versus MspI (circles) as the cumulative percentage of MspI counts stayed within 5 percentage points of EcoRI fragments. The MspI mean distribution of radiolabeled fragments ≥ 4.4 kbp was 27% for both 0.8 and 1.4% agarose (Figure 40) compared to 23% for the MCID scanned gel negatives (Figure 39). The 2.0 kbp markers correspond to 42 - 39% for radiolabeled DNA digested with MspI (Figure 40) and to 46% of the cumulative OD (Figure 39). Both of these disparate methods of analysis indicated that a significant number of MspI fragments from C. albicans have electrophoretic sizes larger than expected.

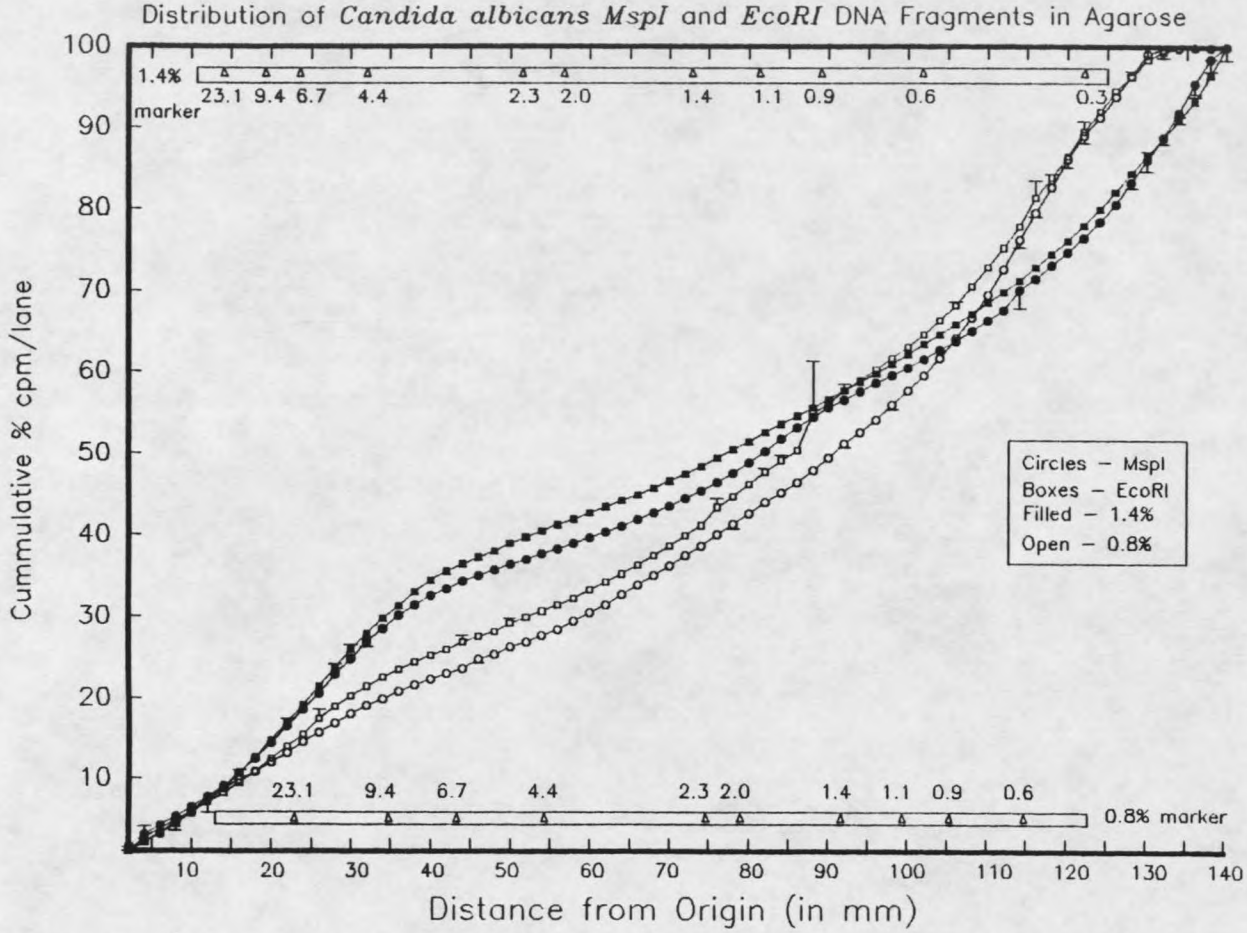


Figure 40. Distribution of MspI and EcoRI ^{32}P-DNA fragments. 0.8 and 1.4% TAE agarose gels were electrophoresed to separate four lanes of MspI or EcoRI digested C. albicans 9938 DNA. 70x2mm slices for each lane were counted, expressed as percent of total cpm/lane, and the cumulative mean for each enzyme (MspI - circles, EcoRI - boxes) graphed as open symbols (0.8% gel) or filled symbols (1.4% gel). Lambda HindIII and ϕX174 HaeIII marker lanes were EtBr stained from each gel and the migration distance was measured and marked on the graph.

APPENDIX C

NUCLEAR DNA ISOLATION METHODS

NUCLEAR DNA ISOLATION METHODS

Efforts to obtain purified nuclear DNA from C. albicans have so far been unsuccessful. The only literature report describes attempts using standard CsCl:EtBr gradient centrifugation (separating DNA based on G+C% differences and circular versus linear form) and differential centrifugation for separating intact organelles (238). They reported G+C values for purified mtDNA (38.2% G+C) and enriched (but impure) nuclear DNA (32.8% G+C) but were unable to isolate separate bands from the CsCl gradients. Intact mitochondria were purified by differential centrifugation but pure nuclear fractions were not obtained. Mitochondrial DNA (mtDNA) has been used as an RFLP biotyping tool to probe EcoRI-digests of C. albicans isolates (245) because mtDNA usually yields six EcoRI fragments that can be distinguished from background nuclear DNA contamination.

In addition to using the Wills, et al. (238), protocol for crude nuclear preparation, I tested two other procedures for DNA or organelle fractionation (successful for S. cerevisiae, S. carlsbergensis, and Kluyveromyces fragilis) and tracked succinate dehydrogenase activity to indicate contamination by functional mitochondria (397).

CsCl:Bisbenzimide Gradients

Spheroplasts of C. albicans B311 and S. cerevisiae ATCC 9896 were prepared as described in the methods section, and processed as described

by Hudspeth, et al. (305), with a few modifications. Spheroplasts (10-20 g) were suspended in 20 ml ice-cold breakage buffer (0.9 M sorbitol, 10 mM Na_2EDTA, 0.1 M Tris-HCl pH 7.5, 0.1% BSA) and hand-agitated with 100 g sterile, 0.5 mm glass beads for 2-3 min. The combined supernatant fluids from initial decanting and 5 x 50 ml bead washes were centrifuged 1500 x g, 10 min, to pellet nuclei and cell debris. The pellet was treated as nuclei enriched and the supernatant processed as mitochondrial rich fractions.

DNA extraction of the nuclear pellet was done either by the organelle lysis procedure or French Pressure Cell method (post-breakage steps starting with suspension in A-1 buffer followed by deproteinizing and RNAse treatment). Mitochondria were pelleted (15,000 x g, 15 min) out of the supernatant fluid, suspended in 100 ml breakage buffer (minus the BSA), and recentrifuged at 20,000 x g, 20 min. The mitochondria were lysed in 7.5 ml of 10 mM Tris-HCl pH 8.2, 10 mM Na_2EDTA, 100 mM NaCl, plus 2% sarkosyl for 2 h. The lysate was EtOH ppt, RNAse treated, and EPR and SEVAG extracted to remove proteins.

Extracts of the nuclear and mitochondrial fractions were suspended in 10 mM Tris-HCl pH 7.5, 20 mM NaCl, 1 mM Na_2EDTA; CsCl was added to a density of 1.693 g/ml (adjusted using a refractometer) and bisbenzimide to 100 µg/ml (Hoechst 33258, Aldrich Chemical Co., Milwaukee, WI). Samples were centrifuged in a Sorvall vertical rotor (TV865B) for 20 h at 20^0 C and bands illuminated with long UV.

Only one band was observed in each of the C. albicans gradient tubes indicating that preferential binding of bisbenzimide to A-T rich regions was unable to provide sufficient enhancement to separate mtDNA

and nuclear DNA (data not shown). DNA from each tube when digested with EcoRI and MspI indicated cross contamination (data not shown).

Ficoll Differential Centrifugation

A method by Wintersberger, et al. (398) involving differential centrifugation in ficoll, followed by discontinuous ficoll gradient step (28,399) was tested on C. albicans 1 strain. Spheroplasts were osmotically lysed in 18% (w/v) ficoll in 0.02 M KH_2PO_4 pH 6.8, 0.5 mM $MgCl_2$, Dounce homogenized and centrifuged twice at 3,000 x g, 5 min, to remove unbroken spheroplasts. The supernatant fluid was centrifuged at 30,000 x g, 20 min, to pellet nuclei (mitochondria and vacuoles should float to the top (398)). The resulting pellet was suspended in the above ficoll solution and centrifuged again. The second pellet was suspended (1 g pellet/ 10 ml) in the 18% ficoll solution and layered onto 30% (w/v) ficoll in 0.02 M KH_2PO_4 pH 6.5 and centrifuged 100,000 x g, 45 min (SW41Ti, 28.5K) to pellet nuclei. Succinate dehydrogenase (SDH) testing (400) for the presence of functional mitochondria was done on some of the fractions. The Lowry (Folin-Ciocalteu) protein assay (204,401) with BSA as a standard was used to assess protein content of the fractions. Figure 41 includes a flow chart of fraction collection and the SDH activity for tested fractions.

DNA extracted from the various gradient fractions (by organelle lysis or cellular DNA extraction methods) were electrophoresed (Figure 42). The samples in Figure 42 indicate extensive shearing of nuclear DNA in many fractions (lanes 1, 7, 10, 14, 22, 28, 31 and 35). Even though only a few samples were successfully digested by EcoRI, the

presence of large amounts of sheared DNA suggests that neither the buffered ficoll or sucrose lysis procedures osmotically protect nuclei of C. albicans. The SDH activity in some pellets indicates that some mitochondria partition at higher density than expected. In fact, the MtD organelles when layered onto the 30% (w/v) ficoll step gradient pelleted to the bottom (data not shown).

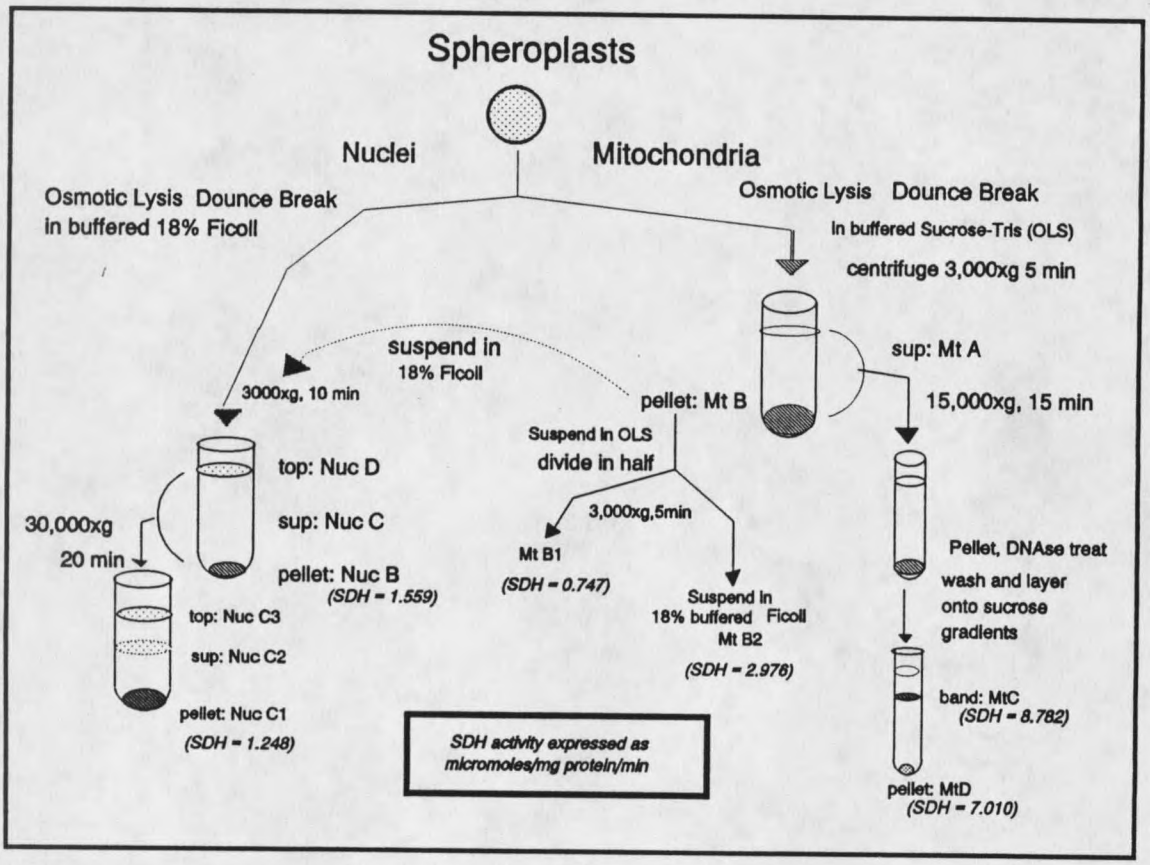


Figure 41. Flow chart of gradient fractions

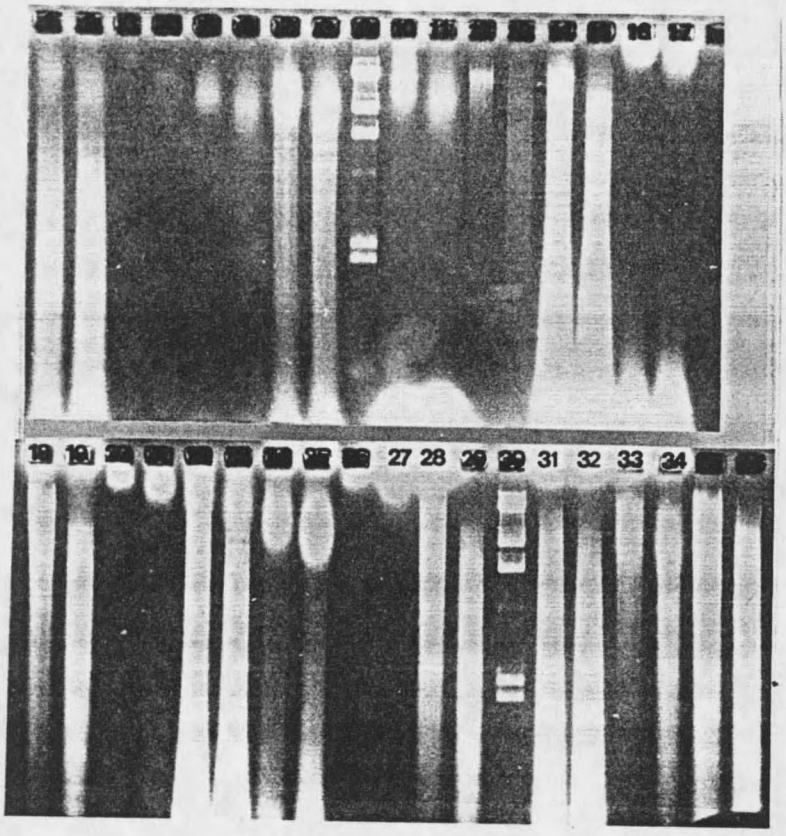


Figure 42. DNA isolated from gradient fractions. C. albicans 1 strain was grown in YEPL (contains lactic acid) or GYEP and DNA from various cell fractions (diagrammed in Figure 41) was RNAse and proteinase K treated. The DNA samples were loaded in pairs for electrophoresis on 1% TAE agarose gels, the first lane contained undigested DNA and the second lane of each pair was EcoRI digested. DNA in lanes 1-8 were from YEPL cultures and the rest of the lanes are from GYEP log phase cultures. Top Gel: Lanes 1 and 2 - MtC; Lanes 3 and 4 - MtD; Lanes 5 and 6 - MtB pelleted through 30% Ficoll, Lane 7 and 8 - MtB interface from 18/30% step gradient; Lane 9 - Lambda HindIII marker; Lanes 10 and 11 - NucC1; Lanes 12 and 13 - NucC2; Lanes 14 and 15 - NucC3; Lanes 16 and 17- NucA. Bottom Gel: Lanes 18 and 19 - OLS whole cell homogenate; Lanes 20 and 21 - MtA; Lanes 22 and 23 - MtB1; Lanes 24 and 25 - MtB2; Lanes 26 and 27 - MtC (sample was re-ppt and the mtDNA digested fine on later gels); Lanes 28 and 29 - MtD; Lane 30 - Lambda HindIII marker, Lanes 31 and 32 - NucB; Lanes 33 and 34 - NucC; and Lanes 35 and 36 - NucD.

Fractions NucB, NucC3, NucD, MtB, and MtD contained sheared nuclear DNA that would be impractical for restriction polymorphism comparisons. SDH activity was not assessed for NucC3 or NucD, but the other fractions containing nuclear DNA were contaminated with functional mitochondria.

Though the fundamental methods for obtaining yeast nuclei have existed for thirty years [reviewed in (28)], subsequent work suggests significant modification when applied to different yeasts (398,399). C. albicans appears to be no exception as isolation of intact nuclei is complicated by "heavy" mitochondria. Other methods, such as Percoll (82), Dextran (399), or adjustment of cation concentration, may provide the necessary nuclear stability to obtain morphologically intact nuclei for high molecular weight nuclear DNA from C. albicans.

APPENDIX D

CULTURE DENSITY VERSUS KLETT VALUES

CULTURE DENSITY VERSUS KLETT VALUES

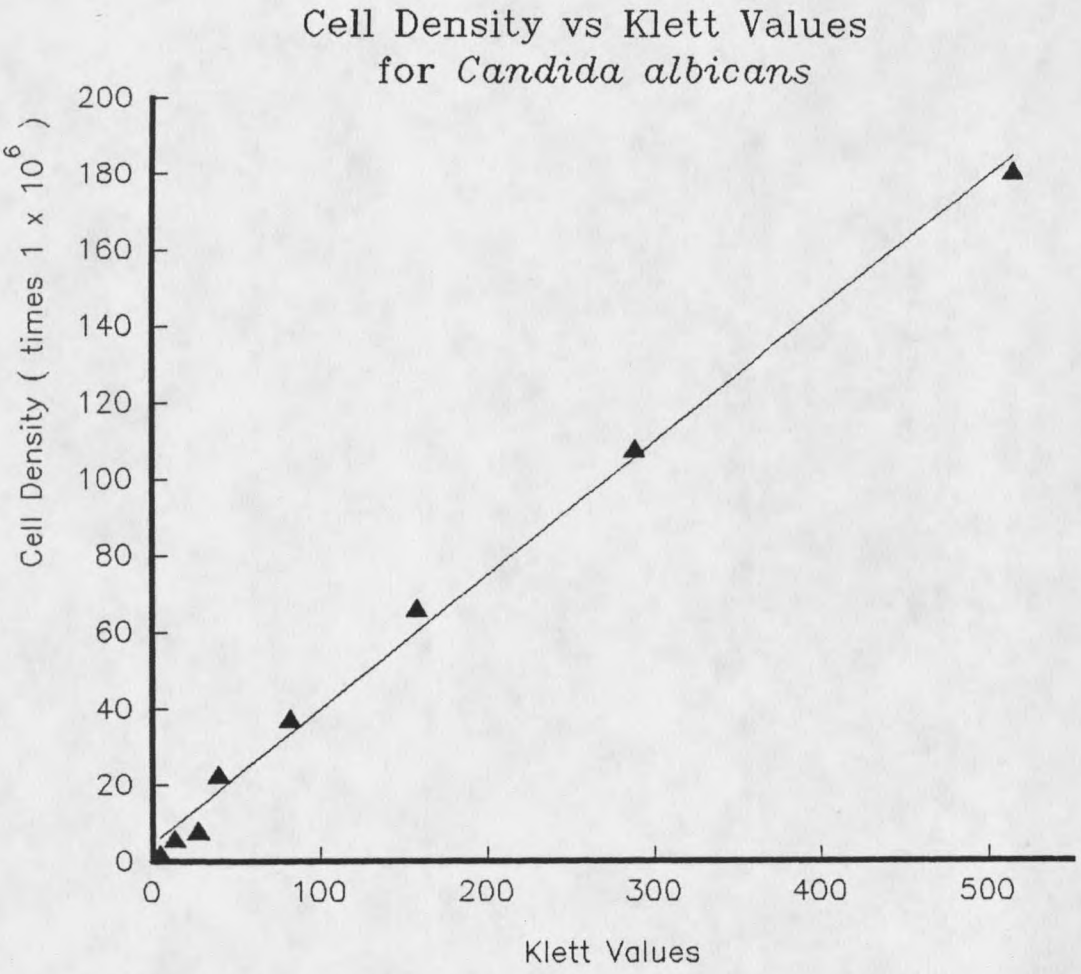


Figure 43. Culture density versus Klett values. A C. albicans B311 stationary phase culture was diluted and 5 ml samples (in 13 x 100 mm test tubes) were placed in a calibrated Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., Inc., New York) fitted with a No. 66 filter. Cell density per ml of sample was determined with a Spencer Bright-line improved Neubauer hemocytometer (American Optical Scientific Instrument Division, Buffalo, NY) and plotted against Klett absorbance values by using Sigmaplot (Figure 43). Cell density divisions on the y axis represent the value multiplied by 1 x 10^6 cells/ ml.

The linear regression line represents the approximate correlation between Klett absorbance and culture density. Klett data has limited potential to reflect actual cell number because different isolates of C. albicans may vary in cell size. Therefore, the graph was intended for approximate estimates and only used during this work for estimating general growth of cultures.

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