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Morphogenesis of *Candida albicans* and Cytoplasmic Proteins Associated with Differences in Morphology, Strain, or Temperature

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The extent of change in cytoplasmic proteins which accompanies yeast-to-mycelium morphogenesis of *Candida albicans* was analyzed by two-dimensional gel electrophoresis. Pure cultures of yeasts and true hyphae (i.e., without concomitant production of pseudohyphae) were grown in a synthetic low-sulfate medium. The two strains selected for this study were strain 4918, which produces pure mycelial cultures in low-sulfate medium at 37°C and yeast cells at 24°C, and strain 2252, which produces yeast cells exclusively at both 24 and 37°C in low-sulfate medium. The proteins of both strains were labeled at both temperatures with [^{35}S]sulfate, cytoplasmic fractions were prepared by mechanical disruption and ultracentrifugation, and the labeled proteins were analyzed by two-dimensional electrophoresis. Highly reproducible protein spot patterns were obtained which defined hundreds of proteins in each extract. Ten protein spots were identified on the two-dimensional gels of the 4918 mycelial-phase extract which were not present in the 4918 yeast-phase extract. These proteins appeared to be modifications of preexisting yeast-phase proteins rather than proteins synthesized de novo in the mycelial cells because 5 were absorbed by rabbit anti-yeast-phase immunoglobulin and each of the 10 was also present in extracts of strain 2252 grown at 24 and 37°C, indicating that they were neither unique to filamentous cells nor sufficient for induction or maintenance of the mycelial morphology. Thirty-three proteins were identified in the 4918 yeast-phase extract which were not present in the 4918 mycelial-phase extract. Pulse-chase experiments revealed the synthesis of new proteins during yeast-to-mycelial conversion, but none of these was unique to mycelial cells. No differences in the major cytoplasmic proteins of any of the yeast- or mycelial-phase extracts were identified. This finding suggests that the major structural proteins of the cytoplasm are not extensively modified and argues instead that proteins unique to either phase may serve a regulatory function.

Candida albicans is a dimorphic fungus able to grow in yeast and mycelial forms. The organism normally exists as a benign member of the mammalian microbial flora, but it can cause life-threatening disease in a compromised host. Under in vivo conditions, the mycelial form is expressed only during invasive colonization, yet little is known of the molecular basis of yeast-to-mycelial morphogenesis (27). Dimorphism is relevant not only to the pathogenicity of *C. albicans* and to the clinical problems of diagnosis and treatment of candidiasis, but also to a basic understanding of mechanisms of morphogenesis in fungi and other eucaryotic cells.

The morphological spectrum of *C. albicans* includes yeast cells, true hyphae, and pseudohyphae. Pseudohyphae consist of chains of elongated yeast cells; they are distinctly different from true hyphae or mycelial cells in appearance and in the composition of their cell walls and septa (11, 13, 14, 21, 32).

Several experimental approaches can be and have been applied to the investigation of morphogenesis in *C. albicans*. The use of a filamentous mutant by Nickerson and Chung supports the theory that disulfide bonds in the cell wall are important in maintenance of the mycelial morphology (26). Many studies have attempted to define a single environmental stimulus that would trigger morphogenetic conversion. Overall, the results of these experiments have been confusing and contradictory (27). Furthermore, the existence of strain variation argues against

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a single, universal stimulus. Selective inhibitors of macromolecular synthesis have been tested for their ability to block morphogenesis. The problems to date with these experiments have been the resistance (or impermeability) of *C. albicans* to the inhibitor(s) tested or the lethality of the inhibitor (29; M. Manning, Ph.D. thesis, Duke University, Durham, N.C., 1979). Biochemical comparisons of membranes, walls, and wall synthetic enzymes between yeast and filamentous cells have not revealed absolute or striking differences (4, 6, 7, 23-25). Unfortunately, the media or conditions used in these studies to grow each morphological form were often complex or very different, or the "mycelial" preparations were contaminated with pseudohyphae or yeasts, or a combination of these factors affected the results. (The membrane and wall composition of *C. albicans* can be affected by medium [31]). Uptake and incorporation of radioactive nucleotides and amino acids follow similar cyclic patterns in synchronous cultures of yeast and mycelial cells (8, 9). Hyphal formation appears to be independent of DNA synthesis, as both DNA synthesis and nuclear migration occur after emergence of the germ tube (34). However, RNA synthesis does seem to vary with environmental conditions and has been variously reported to increase or decrease during morphogenesis (8, 15, 16, 36, 37).

Detailed analyses of the macromolecular differences between the two morphological phases have been few in number. Dabrowa et al. (8) analyzed a cytoplasmic fraction containing fewer than 12 proteins by polyacrylamide gel electrophoresis and described one protein specific to the yeast phase and two associated only with filaments. Cytoplasmic extracts have also revealed the presence of antigens unique to each phase (10, 35).

The investigation described here analyzed the differences in cytoplasmic proteins between yeast and mycelial cells of *C. albicans*. Using a previously described low-sulfate synthetic medium (LSM), we selected two strains, designated 2252 and 4918, for study (22). Both strains produce yeast cells exclusively in LSM at 24°C, but at 37°C strain 4918 develops pure mycelia and strain 2252 develops only yeast cells. By analyzing the cytoplasmic proteins of both strains grown at both temperatures, it was possible to control for protein changes attributable to the strain or temperature alone and not dependent on morphology. The O'Farrell two-dimensional gel technique (28) was used because the protein population of each cell type could be directly visualized and compared. Moreover, by combining this technique with pulse-chase and antibody cross-absorption experiments, it was possible to determine not only protein changes that were unique to each phase, but also whether those changes were due to de novo protein synthesis or to modifications of preexisting proteins.

MATERIALS AND METHODS

Cultures. *C. albicans* strains 4918 and 2252 were isolated from patients with systemic candidiasis at Duke and Minnesota University Hospitals, respectively (22). They were maintained by fortnightly transfers onto a slant medium containing 2% (wt/vol) glucose, 1% yeast extract (Difco Laboratories, Detroit, Mich.), and 2% agar (GYE). To safeguard against spontaneous mutation, multiple slants of each strain were sealed and stored at −20°C.

Growth conditions. For cultivation of yeast- and mycelial-phase cells, LSM, composed of glucose, salts, and biotin, was prepared, and cultures were established as described previously (22). Briefly, cells from a 48-h GYE slant were washed in sterile, deionized water and grown in LSM overnight at room temperature on a rotary shaker (model G76, New Brunswick Scientific Co., New Brunswick, N.J.) at 150 rpm. By this time, the cells had reached early stationary phase and a population of 2×10^8 to 3×10^8 yeast cells per ml with <5% budding forms as determined by microscopic count in a hemacytometer chamber. The cells were then washed and resuspended in fresh LSM, and cultures were established at 1×10^7 to 5×10^7 yeast cells per ml in 200-ml volumes in 500-ml Erlenmeyer flasks. Replicate LSM culture flasks were incubated in rotary water-bath shakers at 150 rpm for 3 h at 37°C and for 6 h at 24°C. Under these conditions, comparable growth and incorporation of [^{35}S]sulfate were achieved by both strains at both temperatures; however, strain 4918 produced 100% true mycelial cells at 37°C and exclusively yeast cells at 24°C, whereas strain 2252 produced only yeast cells at both temperatures.

Cytoplasmic extracts. Both strains of *C. albicans* were grown in LSM with $Na_2{}^{35}SO_4$ as described above, and cells were harvested after 3 h at 37°C or 6 h at 24°C. Cultures were removed immediately to a salted ice bath (−4°C), placed in chilled centrifuge bottles, harvested at 10,000 × *g* at 4°C for 30 min, and washed twice in ice-cold 40 mM sodium phosphate buffer, pH 7.2, with 5 mM EDTA (PBE). The final pellet was resuspended in cold PBE with 1.0 mM phenylmethylsulfonyl fluoride to minimize autoproteolysis (30). The cells were transferred to preweighed Braun homogenizer bottles (or to 7-ml Vacutainer tubes for low-volume runs) in a salted ice bath and were broken by mechanical disruption with glass beads in an MSK cell homogenizer (B. Braun, Inc., San Mateo, Calif.) for 90 to 120 s under pulses of liquid CO_2 for cooling until microscopic examination revealed fewer than five intact cells per high-power field. Glass beads, unbroken cells, and cell debris were removed by centrifugation at 4°C or by filtration on ice. The supernatant or filtrate was then centrifuged at 75,000 × *g* at 4°C for 60 min, and the supernatant was stored in small volumes at −70°C. The extracts were designated 4918-24Y, 4918-37M, 2252-24Y, and 2252-37Y to indicate

strain identification (4918 or 2252), temperature of cultivation (24 or 37°C), and morphology (Y, yeast, or M, mycelium). Similarly prepared extracts contained, as the percentage of dry weight, 20 to 26% protein, 3 to 6% hexose, and ca. 3% nucleic acid (M. Manning and T. G. Mitchell, Infect. Immun., in press).

Labeling proteins with [^{35}S]sulfate. Proteins were radioactively labeled with [^{35}S]sulfate by growing cells in LSM with $Na_2{}^{35}SO_4$. The specific activity of the radionuclide was approximately 1 Ci/mmol (New England Nuclear Corp., Boston, Mass.). Each 200-ml culture received 0.5 mCi; cells were harvested and cytoplasmic extracts were prepared as described above. The specific activity of the protein in the extracts was determined after precipitation of the extracts with trichloroacetic acid. Triplicate samples of 0.01 ml each were incubated in 1.0 ml of 5% trichloroacetic acid for 60 min at 4°C. The precipitates were collected on membrane filters, washed successively with ice-cold trichloroacetic acid and 50% ethanol, and dried. Each filter was placed in a liquid scintillation vial, covered with 15 ml of scintillation fluid consisting of 67% (vol/vol) toluene, 33% (vol/vol) Triton X-100, 0.4% (wt/vol) 2,5-diphenyloxazole (PPO), and 0.2% (wt/vol) 1,4-bis-[2]-(5-phenyloxazolyl)benzene (POPOP), and counted in a liquid scintillation counter (model LS-233, Beckman Instruments, Inc., Fullerton, Calif.) with a counting efficiency of 55%. Typically, 70% of the total counts per minute in the cytoplasmic extracts was trichloroacetic acid precipitable, and the specific activity ranged from 3×10^6 to 6×10^6 cpm/mg of protein. Protein concentrations were measured by the method of Lowry et al. (20) with bovine serum albumin used as a standard. Neither growth rate nor morphology of either strain was affected by the supplementation of LSM with $Na_2{}^{35}SO_4$.

Pulse-chase experiments. Yeast-phase cultures of both strains were grown in LSM with $Na_2{}^{35}SO_4$ for 5 h at 24°C. The unincorporated radioisotope was then replaced with the natural isotope by centrifuging the cells at 4°C, washing them twice in a basic salts medium which is rich in unlabeled sulfate (22), and resuspending them at the same concentration in prewarmed basic salts medium supplemented with 1 mM cysteine and 1 mM methionine. The cultures were then grown at 37°C for 45 min and harvested at 4°C, and cytoplasmic extracts were prepared.

Antibody cross-absorption. The methods used for preparation of rabbit hyperimmune antisera to cytoplasmic extracts 4918-24Y and 4918-37M and purified immunoglobulin from pooled antisera and preimmune rabbit sera, as well as the procedure for cross-absorption of the four cytoplasmic extracts with the three immunoglobulin preparations and removal of immune complexes with staphylococcal protein A, are described elsewhere (Manning and Mitchell, in press).

Gel-techniques. The $^{35}SO_4$-cytoplasmic proteins from the four extracts were analyzed by two-dimensional polyacrylamide gel electrophoresis. The procedure used was essentially that described by O'Farrell (28) with the following specific modifications.

(i) Sample. The extracts were not treated with DNase prior to gel analysis. Solid urea was added to each sample to bring the concentration to 9 M urea, and 1 volume of lysis buffer was added.

(ii) First dimension. The first dimension isoelectric focusing gels were made in glass tubing 150 by 2.0 mm (ID). Tubes were filled so that the polymerized gel was 130 mm long. Thirty micrograms of sample protein, in a total volume of less than 50 μl, was applied to the first-dimension isoelectric focusing gel, and electrophoresis was carried out at 400 V for 24 h. The gels were then extruded from the glass tubes into 5 ml of sodium dodecyl sulfate sample buffer and stored at −70°C until the second dimension was run. The pH gradient achieved in gels containing only ampholines of pH range 3 to 10 demonstrated the expected linear pH gradient, but the resolution was enhanced by the addition of ampholines of pH range 5 to 7, which decreased the slope of the gradient from pH 5.5 to pH 7.5 (28). Ampholines were obtained from Bio-Rad Laboratories (Richmond, Calif.).

(iii) Second dimension. A uniform 10% acrylamide lower gel, 130 by 200 by 0.75 mm, was poured with a 30-mm 4% acrylamide upper gel. A piece of Parafilm with a 90° fold was carefully placed on the notch and in contact with the 4% gel, creating a flat gel surface. Prior to electrophoresis in the second dimension, the first-dimension samples were thawed and equilibrated in the sodium dodecyl sulfate sample buffer for 30 min at room temperature with gentle shaking. When the upper gel had polymerized, the Parafilm was removed, molten 1% agarose in sodium dodecyl sulfate sample buffer was pipetted onto the notch, and the equilibrated isoelectric focusing gel was immediately set in place. The top of the gel was sealed with additional agarose. The second-dimension slabs were run at 24 mA until the dye front of bromophenol blue was 5 mm from the bottom of the gel (ca. 4.5 h). The molecular-weight distribution of the proteins in the second-dimension sodium dodecyl sulfate gel ranged from 10,000 to 100,000 as determined from molecular-weight standards (Bio-Rad Laboratories).

(iv) Fixing, drying, and autoradiography. Gels were fixed by soaking overnight in 7.5% acetic acid. They were then dehydrated with several changes of dimethyl sulfoxide, equilibrated with 20% PPO in dimethyl sulfoxide (3), dried (see below), and exposed to preflashed Kodak Rapid Processing X-ray film at −70°C (17). Impregnation with PPO and the use of preflashed film greatly enhanced the sensitivity of the autoradiography. Preflashing also corrects the nonlinear relationship between spot intensity and sample radioactivity (3, 17). It was therefore possible to partially correct for the small differences in the specific activity of the protein in various extracts and to reduce variation in autoradiogram intensity among various gels. Preliminary experiments showed that spot smearing occurred with electrophoresis of samples containing greater than 30 μg of protein (corresponding to 2×10^4 to 6×10^4 cpm). Consequently, long X-ray film exposure times were required to visualize minor protein spots (e.g., the four reference gels and antibody cross-absorption gels were autoradiographed for 6 weeks).

The following apparatus was assembled to expedite the drying of oversized gels. PPO-incorporated gels were carefully placed on a sheet of Whatman 3M chromatography paper (0.33 mm thick) that was cut slightly larger than the gel. Four gels were then arranged on a sheet of porous polyethylene (45 by 45 by 0.64 cm, 34-μm pore size, Bel Arts Products, Pequa-

neck, N.J.) and held in place with household plastic wrap. This polyethylene sheet was then centered, gel side down, on a sheet of Solastic rubber (91.4 by 91.4 by 0.16 cm, Southern Rubber Co., Greensboro, N.C.), covered with a sheet of fine stainless-steel mesh and a second sheet of Solastic rubber which was connected by a vacuum hose to a VirTis Unitrap II lyophilizer. With this apparatus, four O'Farrell gels could be dried simultaneously in 3 to 4 h.

Analysis of two-dimensional gels. In preliminary electrophoresis, O'Farrell gels were observed to create highly reproducible spot patterns of protein migration. The technique was capable of identifying hundreds of individual proteins. However, the patterns are complex and more difficult to evaluate than single-dimension gel patterns. This problem is compounded when many different gels must be analyzed (in this investigation, each gel was compared with nine others). Since slight discrepancies in the length of either the first or second dimension gel may shift the absolute position on the final autoradiogram, the gels cannot be reproduced exactly (i.e., duplicate gels of the same cytoplasmic extract cannot be perfectly superimposed). However, the spots on each gel are highly reproducible in their migration with respect to each other and are easily identifiable because their locations can be defined in relation to nearby spots. Major spot clusters served as both landmarks and internal controls. The following system was devised to maintain a constant coordinate system and to facilitate the comparison of many different gels.

One gel autoradiogram was taped to an illuminated viewing screen over which a plastic transparency sheet was taped, and the corners of this reference gel were demarcated on the transparency. Another gel autoradiogram was then placed beside the reference gel, and spots that were *present* on the reference gel but *absent* from the second gel were denoted on the transparency. The transparency and the second gel were then replaced and the process was repeated. Four gels served as references: 4918-24Y, 4918-37M, 2252-24Y, and 2252-37Y. These were compared with one another and with the gels resulting from the pulse-chase and antibody cross-absorption experiments. Thus, four sets of nine transparencies each were created. A "master" transparency was then made for each set by aligning all nine transparencies and recording all the spots on the master. Each spot was assigned a map coordinate and number. A table was composed containing all the spots that were present on the reference gel but absent on another gel and indicating the difference in conditions between them. To check the reproducibility of this technique, we coded representative gels and had them analyzed independently by another researcher. This method permitted a comprehensive evaluation of the spots, simplified the analysis of very complex protein migration patterns, and allowed simultaneous comparison of the effects of several different experimental conditions. Cytoplasmic protein modifications associated with temperature, strain, or morphogenesis were identified.

RESULTS

Two-dimensional electrophoresis. The two-dimensional gel patterns obtained for 4918-24Y, 4918-37M, 2252-24Y, and 2252-37Y (the four reference gels) are shown in Fig. 1a–d. Each of these extracts was prepared and electrophoresed at least twice. Some spots present on these reference gels were not visible on preliminary gels of similar extracts because earlier autoradiograms were not exposed as long as later ones. Nonetheless, the spots discernible on earlier gels corresponded exactly to spots on the reference gels. No spots were modified or lost. Moreover, the number and arrangement of the satellite spots of the high-molecular-weight proteins (i.e., a closely spaced series of spots of the same molecular weight produced by a protein possessing charge heterogeneity) did not differ. These patterns are thought to result from charge modifications such as phosphorylation or acetylation (28), and their stability indicates that in vitro protein modification is minimal (or at least consistent) in duplicate extracts. Similarly, no spot differences were detectable in duplicate gels of the same extract.

Gel analysis. Two-dimensional electrophoresis of the cytoplasmic extracts of *C. albicans* identified many hundreds of cytoplasmic proteins. The method devised for analysis of the spot patterns of the gels was quite reliable. Four "master" transparencies, which describe the location of each reference gel spot that was absent on another gel, were defined, and the location of each spot was tabulated. Figure 2 and Table 1 present this information for the 4918-37M reference gel. The specific data generated with the other three reference gels are not shown; however, a total of 292 spot changes were noted when reference gel 4918-24Y was compared with the other gels. On the 2252-24Y reference gel, 172 individual proteins were scored, and 232 were scored on 2252-37Y. Table 1 shows exactly which gels lacked each spot. Despite the complexity of the spot patterns, protein changes associated with strain, temperature, or morphology, as well as the results of antibody cross-absorption and pulse-chase experiments, can be readily located. Pulse-chase experiments were undertaken to identify proteins specific to mycelial-phase cells. Yeast-phase proteins of both strains were prelabeled with [^{35}S]sulfate at 24°C, washed, and subsequently cultivated at 37°C in the presence of excess unlabeled sulfate. Mycelial-phase cells were harvested at 45 min rather than 3 h to minimize reutilization of isotope liberated by catabolism of yeast-phase proteins. The results of these experiments are shown in Fig. 3. None of the nine spots missing on the pulse-chase gel was also absent from all three yeast-phase gels (Table 1). These proteins are apparently newly synthesized but not mycelium specific. (Two of these proteins, HJ'e and HJ'g,

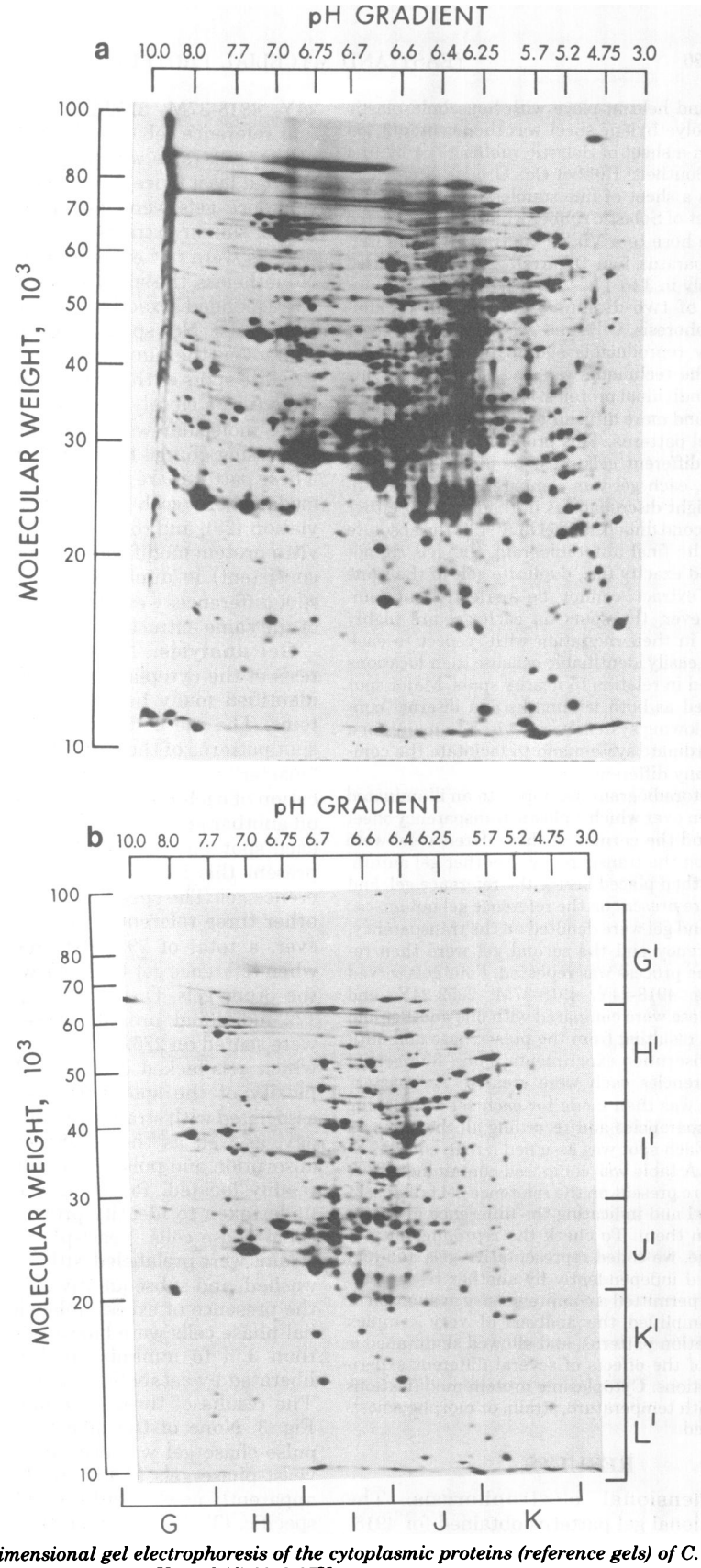
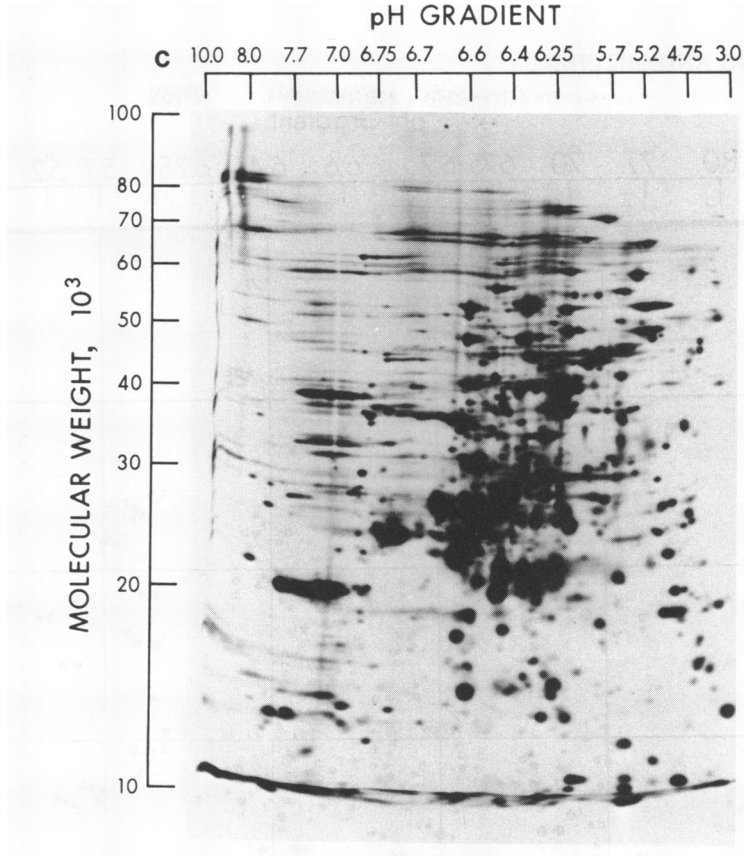


FIG. 1. *Two-dimensional gel electrophoresis of the cytoplasmic proteins (reference gels) of C. albicans: 4918-24Y, (b) 4918-37M, (c) 2252-24Y, and (d) 2252-37Y.*



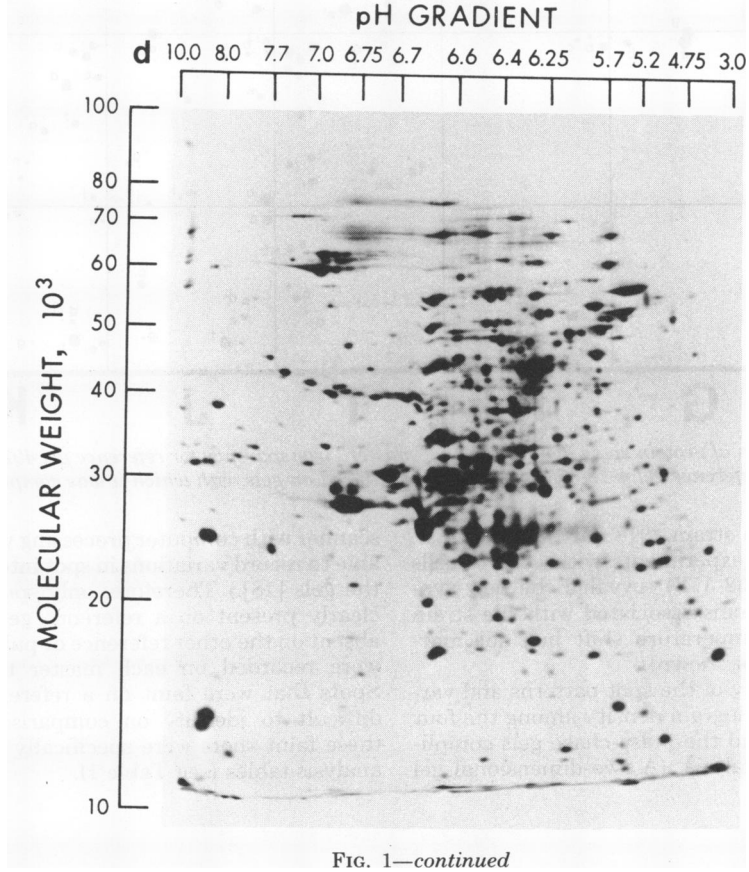


FIG. 1—*continued*

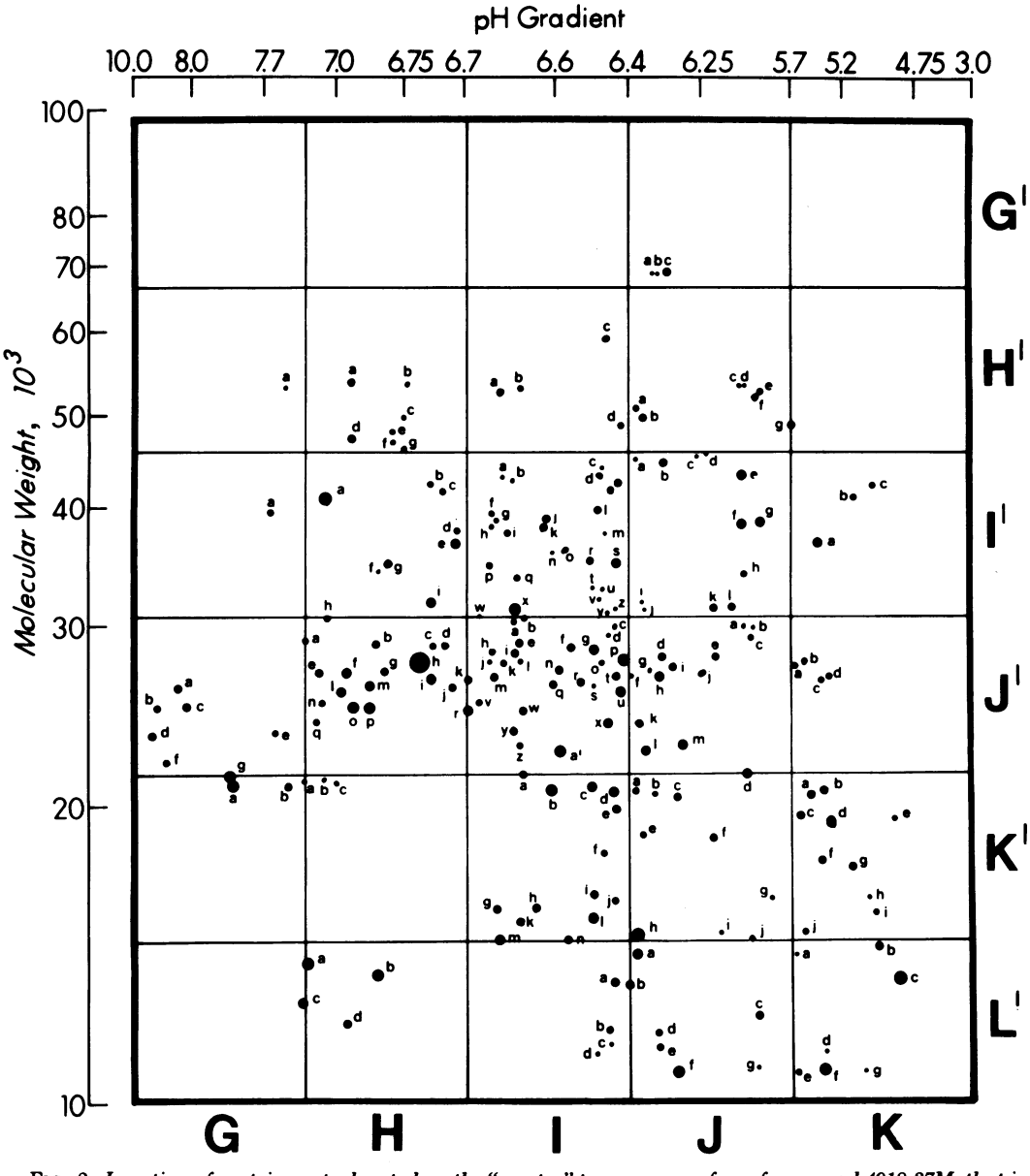


FIG. 2. *Location of protein spots denoted on the "master" transparency for reference gel 4918-37M, that is, spots present on reference gel 4918-37M but absent or reduced on gels with which it was compared.*

were detected in strain 4918 only.) In contrast, the pulse-chase experiments with control cells (2252-24Y → 2252-37Y) revealed the new synthesis of 80 proteins associated with the strain difference or temperature shift but not morphology (data not shown).

The complexity of the spot patterns and variation in autoradiogram density among the four reference gels and the pulse-chase gels complicated the gel analysis. (A two-dimensional gel scanner with computer processing was not available to record variations in spot intensity among the gels [18].) Therefore, only spots that were clearly present on a reference gel and totally absent on the other reference or pulse-chase gels were recorded on each master transparency. Spots that were faint on a reference gel were difficult to identify on comparison gels, and these faint spots were specifically noted in the analysis tables (see Table 1).

TABLE 1. *Analysis of the differences between the protein spot pattern of 4918-37M and the pattern generated by eight other conditions*[a]

4918-37M protein spot[b] vs.:	4918-24Y	2252-24Y	2252-37Y	Pulse-chase	4918-37M absorbed with:[c]			
					α-4918-24Y	α-4918-37M	NRI	SA
JG'a	+	+	0	+	+	+	+	+
b	+	+	0	+	+	+	+	+
c	+	+	0	+	+	+	+	+
GH'a	+	+	+	+	−	+	+	+
HH'a	+	+	+	+	+	−	+	+
b	+	+	+	+	+	−	+	+
c°	+	+	+	+	+	−	+	+
d	+	0	0	+	+	−	+	+
e°	+	+	+	+	+	−	+	+
f°	+	+	0	+	+	−	+	+
g°	+	+	+	+	+	−	+	+
IH'a	+	+	+	+	−	+	+	+
b	+	+	0	+	−	+	+	+
c	+	+	+	+	+	−	+	+
d	+	0	+	+	+	+	+	+
JH'a	+	+	+	+	+	−	+	+
b	+	+	+	+	+	−	+	+
c°	+	+	0	+	+	+	+	+
d°	+	+	0	+	+	−	+	+
e°	+	+	+	+	+	−	+	+
f	+	+	+	+	+	−	+	+
g°	0	+	+	+	+	+	+	+
GI'a	0	+	+	+	−	−	+	+
HI'a	+	0	0	+	−	+	+	+
b	+	+	+	+	−	−	+	+
c	+	+	+	+	−	−	+	+
d	+	+	+	+	+	−	+	+
e	+	+	+	+	+	−	+	+
f°	+	+	+	+	+	−	+	+
g°	+	+	+	+	+	−	+	+
h	+	+	+	+	+	−	+	+
i°	+	+	+	+	+	+	−	+
II'a	+	+	+	+	+	−	+	+
b	+	+	+	+	+	−	+	+
c	+	+	0	+	+	+	+	+
d	+	0	0	+	+	+	+	+
e	+	+	+	0	+	+	+	+
f	+	+	+	+	+	−	+	+
g	+	+	+	+	+	−	+	+
h	+	+	+	+	+	−	+	+
i	+	+	+	+	+	−	+	+
j	+	0	+	+	+	−	+	+
k	+	+	0	+	+	+	+	+
l	+	+	+	+	−	+	+	+
m	+	+	+	+	−	+	+	+
n°	+	+	+	+	+	−	+	+
o°	+	+	+	+	+	−	+	+
p	+	+	+	+	+	−	+	+
q	0	+	+	+	+	−	+	+
r	+	0	+	+	+	−	+	+
s	+	+	+	0	+	+	+	+
t	+	+	+	+	+	−	+	+
u	+	+	+	+	+	−	+	+
v	+	+	+	+	+	−	+	+
w	+	+	+	+	+	−	+	+

TABLE 1—*continued*

4918-37M protein spot[b] vs.:	4918-24Y	2252-24Y	2252-37Y	Pulse-chase	4918-37M absorbed with:[c]			
					α-4918-24Y	α-4918-37M	NRI	SA
x	+	+	+	+	+	−	+	+
y	+	+	+	+	+	−	+	+
z	+	+	+	+	+	−	+	+
JI'a	+	+	+	+	+	−	+	+
b	+	+	0	+	+	+	+	+
c	+	+	+	+	+	−	+	+
d	+	+	+	+	+	−	+	+
e	+	+	+	+	−	−	+	+
f	+	+	+	+	+	−	+	+
g	+	+	+	0	−	+	+	+
h	+	+	+	0	+	−	+	+
i	+	+	+	+	+	−	+	+
j	+	+	+	+	+	−	+	+
k	+	+	0	+	+	+	+	+
l	+	+	0	+	+	+	+	+
KI'a	+	+	0	+	−	+	+	+
b°	+	+	+	+	+	−	+	+
c°	+	+	+	+	+	−	+	+
GJ'a°	+	0	+	+	+	+	+	+
b°	+	0	+	+	+	+'+	+	+
c°	+	0	+	+	+	−	+	+
d°	+	+	+	+	+	+	+	+
e	+	+	+	+	−	−	+	+
f°	+	+	+	+	+	−	+	+
g	+	+	+	+	+		+	+
HJ'a	+	+	+	+	+	−	+	+
b	+	+	+	+	+	−	+	+
c	0	+	+	+	−	−	+	+
d	0	+	+	+	−	−	−	+
e	+	0	0	0	−	−	+	+
f	+	+	0	+	−	+	+	+
g	+	0	0	0	+	+	+	+
h	+	+	+	+	−	−	+	+
i	+	+	+	0	−	−	+	+
j	+	+	+	+	+	−	−	+
k	+	+	+	+	−	−	+	+
l	0	+	+	+	−	−	+	+
m	+	+	+	0	−	+	+	+
n	+	+	+	+	−	+	+	+
o	+	+	+	+	−	−	+	+
p	+	+	+	+	−	−	+	+
q	+	+	+	+	−	+	+	+
r	+	+	+	+	−	−	−	+
IJ'a	0	+	0	+	+	−	+	+
b	0	+	0	+	+	−	+	+
c	+	+	+	+	+	−	+	+
d	+	+	+	+	+	−	+	+
e	+	+	+	+	−	−	+	+
f	+	+	+	+	−	−	+	+
g	+	+	+	+	−	−	+	+
h	+	+	+	+	−	+	+	+
i	+	+	+	+	+	−	+	+
j	+	+	+	+	−	+	+	+
k	+	+	+	+	+	−	+	+
l	+	+	+	+	+	−	+	+
m	+	+	+	+	−	+	+	+
n	+	+	+	+	+	−	+	+
o	+	+	+	+	−	−	+	+
p	+	+	+	+	−	−	+	+
q	+	+	+	+	+	−	+	+
r	+	+	+	+	−	−	+	+

TABLE 1—*continued*

4918-37M protein spot[b] vs.:	4918-24Y	2252-24Y	2252-37Y	Pulse-chase	4918-37M absorbed with:[c]			
					α-4918-24Y	α-4918-37M	NRI	SA
s	+	+	+	+	−	−	+	+
t	+	+	+	+	−	+	+	+
u	+	+	+	+	−	+	+	+
v	+	+	+	+	−	−	+	+
w	+	+	+	+	−	−	+	+
x	+	+	0	+	+	+	+	+
y	+	+	+	+	−	−	+	+
z	+	+	+	+	−	−	+	+
a′	+	+	+	+	+	−	+	+
JJ′a°	+	0	+	+	+	−	+	+
b°	+	0	0	+	+	−	+	+
c°	+	0	0	+	+	−	+	+
d	0	+	+	+	+	−	+	+
e	+	+	0	+	+	−	+	+
f	+	+	+	+	−	+	+	+
g	+	+	+	0	+	−	+	+
h	+	+	+	+	+	−	+	+
i	+	+	+	+	+	+	−	+
j	+	+	+	+	+	+	+	+
k°	+	+	0	+	+	+	+	+
l	+	+	+	+	+	−	+	+
m	+	+	+	+	+	−	+	+
KJ′a	+	+	+	+	−	−	+	+
b	+	+	+	+	−	+	+	+
c	+	+	+	+	−	+	+	+
d	+	+	+	+	−	+	+	+
GK′a	+	+	+	+	+	−	+	+
b°	+	+	+	+	+	−	+	+
HK′a°	+	+	+	+	+	−	+	+
b	+	+	+	+	+	−	+	+
c	+	+	+	+	+	−	+	+
IK′a	+	+	+	+	−	+	+	+
b	+	+	+	+	−	−	+	+
c	+	+	+	+	−	−	+	+
d	+	+	+	+	−	−	+	+
e	+	+	+	+	−	−	+	+
f	+	+	+	+	+	−	+	+
g	+	+	+	+	−	−	+	+
h	+	0	+	+	+	−	+	+
i	+	+	+	+	−	−	+	+
j	+	+	+	+	−	−	+	+
k	+	0	+	+	−	−	+	+
l	0	+	+	+	−	+	+	+
m	+	+	+	+	−	+	+	+
n	+	+	+	+	−	+	+	+
JK′a	+	+	+	+	−	−	+	+
b°	+	+	+	+	−	−	+	+
c	+	+	+	+	−	−	+	+
d	+	+	+	+	+	−	+	+
e	+	+	+	+	+	−	+	+
f	+	+	+	+	+	−	+	+
g°	+	+	+	+	−	+	+	+
h	+	+	+	+	+	−	+	+
i°	+	+	+	+	−	+	+	+
j°	+	+	+	+	−	+	+	+
KK′a	+	+	+	+	−	−	+	+
b	+	+	+	+	−	−	+	+
c	+	+	+	+	−	−	+	+
d	+	+	+	+	−	−	+	+
e	+	+	+	+	−	+	+	+

TABLE 1—*continued*

4918-37M protein spot[b] vs.:	4918-24Y	2252-24Y	2252-37Y	Pulse-chase	4918-37M absorbed with:[c]			
					α-4918-24Y	α-4918-37M	NRI	SA
f°	+	+	+	+	−	−	+	+
g	+	+	+	+	−	−	+	+
h	+	+	+	+	−	−	+	+
i	+	+	+	+	−	−	+	+
j	+	+	+	+	−	+	+	+
HL'a	+	+	0	+	−	−	+	+
b	+	+	+	+	−	−	+	+
c	+	+	0	+	−	−	+	+
d	+	+	+	+	−	−	+	+
IL'a	+	+	+	+	−	−	+	+
b°	+	+	+	+	−	+	+	+
c°	+	+	+	+	−	−	+	N
JL'a	+	+	+	+	+	−	+	+
b	+	+	+	+	−	−	+	+
c	+	+	+	+	+	−	+	+
d°	+	+	+	+	+	+	+	N
e°	+	+	+	+	+	+	+	N
f°	+	+	+	+	+	+	+	N
g	+	+	+	+	−	+	+	N
KL'a°	+	+	+	+	+	+	+	+
b	+	+	+	+	−	−	+	N
c	+	+	+	+	−	+	+	N
d	+	+	+	+	+	+	+	N
e	+	+	+	+	+	+	+	N
f	+	+	+	+	+	+	+	N
g°	+	+	+	+	+	+	+	N

[a] Symbols: +, spot present on both 4918-37M and any other compared gel; 0, spot present on 4918-37M but absent from another reference or pulse-chase gel; −, spot present on 4918-37M but absent or significantly decreased on antibody-absorbed gels; N, spot not identified because of smearing at gel edge.

[b] Protein spot denotes the location of the spot on the master transparency shown in Fig. 2; the sign ° indicates a very faint spot.

[c] α-4918-24Y, anti-4918-24Y immunoglobulin; α-4918-37M, anti-4918-37M immunoglobulin; NRI, normal rabbit (preimmune) immunoglobulin; SA, *Staphylococcus aureus*.

The autoradiogram density of the reference gels and the corresponding antibody cross-absorption gels was more uniform because the same radiolabeled extract was used for each set. Comparison of spot intensity among these gels was therefore more feasible, and spots significantly reduced by cross-absorption were noted on the master transparencies. Most of the spot changes noted were associated with the cross-absorption gels.

Table 2 presents a compilation of the total number of proteins that were present on each reference gel and absent from one, two, or three of the other three reference gels.

Strain variation. Proteins found in strain 4918 but not in strain 2252 were identified by their absence on both 2252 reference gels and their concomitant presence on the 4918 reference gels. At 24°C, strain 4918 contained 16 such spots (Table 2), and at 37°C, strain 4918 displayed 7 strain-specific proteins (e.g., Table 1, HH'd). The approximate molecular weight and pI of each protein scored on 4918-37M are evident from Fig. 2. For example II'j had a molecular weight of approximately 40,000 and an isoelectric point near 6.6. Protein spots found only in strain 2252 were identified in a similar manner. Table 2 indicates 14 and 16 spots not present in strain 4918 when compared with the 2252 reference gels. Interestingly, 4 of these spots were absorbed by either or both anti-4918-24Y and anti-4918-37M, indicating antigenic similarity to proteins in strain 4918 (data not shown).

Temperature. Only two proteins were present in both strains cultivated at 37°C but absent from both grown at 24°C (Table 2). Fifteen proteins were revealed only at 24°C.

Morphology. Tables 1 and 2 indicate that a total of 10 protein spots are present in the mycelial phase of strain 4918 but absent in the yeast phase of the same strain (e.g., HJ'c, IJ'a). These proteins are possibly associated with filamentation. Further examination of Table 1, however, reveals that none of these same spots was also absent from both 2252-24Y and 2252-37Y, and therefore they are not unique to mycelial-phase

cells. Eight of the 10 spots were removed by absorption with anti-4918-37M, and 5 were removed by anti-4918-24Y (e.g., HJ'l and IK'l). In addition, none of the spots was absent from the 4918 pulse-chase gel, indicating that these "mycelial" proteins may, in fact, constitute modified yeast-phase proteins.

Analysis of the yeast-phase reference gels revealed many proteins which were present in all three yeast cell extracts but absent in the mycelial-phase extract. The yeast phase of strain 4918 contained 33 proteins not evident in 4918-37M (Table 2). Only two of these were removed by cross-absorption with the anti-mycelial-phase immunoglobulin. Strain 2252 at 24 and 37°C had 50 and 53 (Table 2) cytoplasmic proteins, respectively, which were absent only on reference gel 4918-37M.

DISCUSSION

By examining the cytoplasmic proteins of yeast and mycelial cells, we have begun to define the extent of the macromolecular change involved in morphogenesis of *Candida albicans*. Furthermore, techniques have been developed for extracting cytoplasmic proteins with a minimum of in vitro modification and for analyzing and comparing the complex O'Farrell two-dimensional gel patterns produced from these extracts. Two-dimensional gel electrophoresis of the cytoplasmic proteins of *C. albicans* strains 4918 and 2252 generated highly reproducible spot patterns, identifying many hundreds of individual proteins. The gel analysis method enabled both the identification of proteins unique to yeast or mycelial cells (and therefore possibly involved in the maintenance of cell morphology) and the simultaneous comparison of the fate of each protein under several different conditions, namely, changes associated with strain, temperature, immunoglobulin absorption, and pulse-chase conditions, singly or in combination.

Differences between the predominant cytoplasmic proteins of the two phases were not

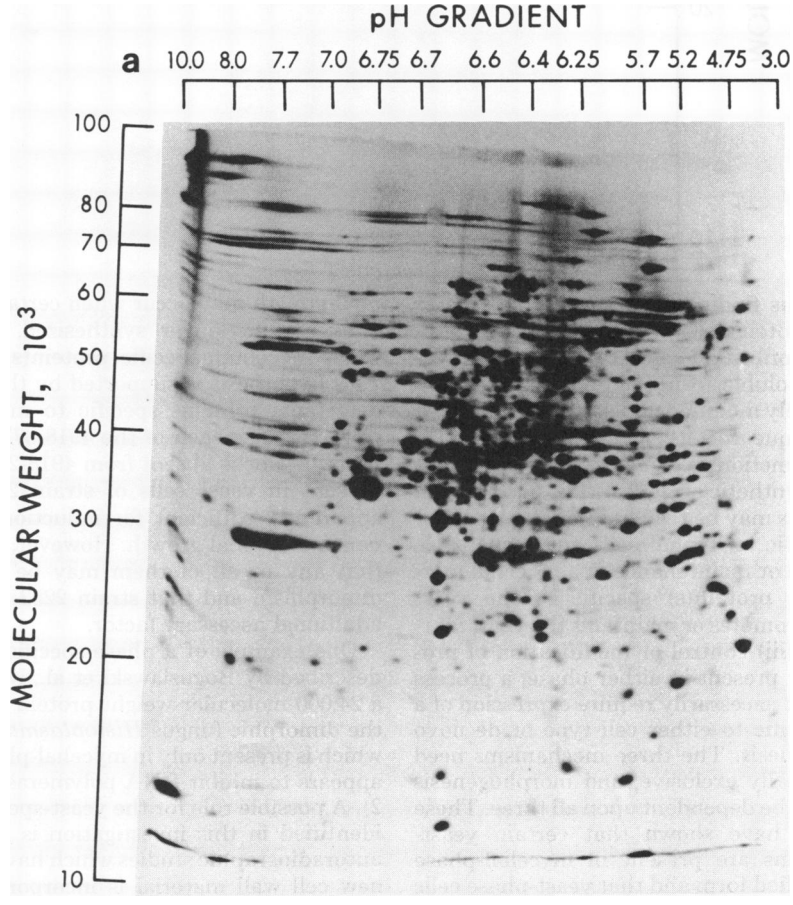


FIG. 3. *Two-dimensional electrophoresis of the cytoplasmic proteins of C. albicans strains (a) 4918 and (b) 2252 under pulse-chase conditions. See text for details.*

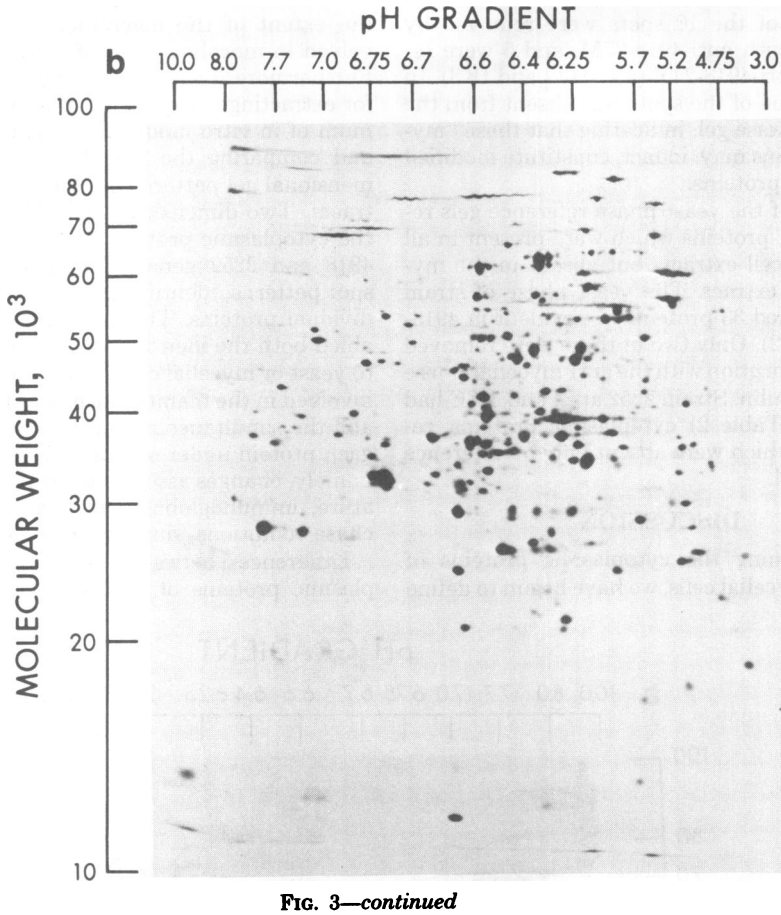


FIG. 3—*continued*

detected. This finding suggests that the major structural proteins of the cytoplasm (e.g., cytoplasmic microfibrils or cell wall components synthesized in soluble form in the cytoplasm) are not extensively modified and argues instead that proteins unique to either phase may serve a regulatory function (e.g., controlling the action of cell wall synthetic enzymes). The dimorphism of *C. albicans* may be regulated by (i) protein(s) that is specific to hyphal cells and responsible for induction or maintenance of a mycelial morphology, (ii) protein(s) specific to the yeast phase that promotes or maintains the yeast morphology, or (iii) control or modification of proteins already present in either phase, a process that does not necessarily require expression of a genome specific to either cell type or de novo protein synthesis. The three mechanisms need not be mutually exclusive, and morphogenesis may actually be dependent upon all three. These experiments have shown that certain yeast-phase proteins are present in mycelial-phase cells in modified form and that yeast-phase cells appear to contain many proteins which are not in mycelial phase cells. Thus, conversion to hyphal growth may occur when certain yeast proteins are no longer synthesized, rather than when mycelium-specific protein(s) is induced. This hypothesis is supported by the absence of detectable proteins specific to mycelial-phase cells. Because each of the 4918-37M associated proteins (those absent from 4918-24Y) was also present in yeast cells of strain 2252, none is apparently sufficient for induction or maintenance of hyphal growth. However, it is possible that any or all of them may be required for dimorphism and that strain 2252 is lacking an additional necessary factor.

One example of a phase-specific protein was described by Boguslawski et al., who identified a 24,000-molecular-weight protein (histin) from the dimorphic fungus *Histoplasma capsulatum* which is present only in mycelial-phase cells and appears to inhibit RNA polymerase activity (1, 2). A possible role for the yeast-specific proteins identified in this investigation is suggested by autoradiographic studies which have shown that new cell wall material is incorporated only at hyphal apices, whereas in yeast cells wall biosynthesis occurs uniformly around the cell (ex-

TABLE 2. *Summary of comparative analysis of the four reference gels*

Absent from:	No. of protein spots present on reference gels:			
	4918-24Y	4918-37M	2252-24Y	2252-37Y
4918-24Y only	—[a]	8	7	9
4918-37M only	28	—	50	53
2252-24Y only	44	9	—	10
2252-37Y only	90	19	31	—
4918-24Y and 4918-37M	—	—	14	16
4918-24Y and 2252-24Y	—	0	—	2
4918-24Y and 2252-37Y	—	2	2	—
4918-37M and 2252-24Y	1	—	—	5
4918-37M and 2252-37Y	2	—	13	—
2252-24Y and 2252-37Y	16	7	—	—
4918-24Y and 4918-37M and 2252-24Y	—	—	—	4
4918-24Y and 4918-37M and 2252-37Y	—	—	13	—
4918-24Y and 2252-24Y and 2252-37Y	—	0	—	—
4918-37M and 2252-24Y and 2252-37Y	2	—	—	—
Total absent from:				
4918-24Y	—	10	36	31
4918-37M	33	—	90	79
2252-24Y	46	16	—	21
2252-37Y	110	28	59	—

[a] —, Not applicable.

cept at bud scar sites), promoting spherical growth (31, 33). Dimorphism, then, may involve either selective activation of wall-associated synthetic enzymes or direction of soluble synthetic enzymes to specific wall sites. In support of this theory, Cabib (5) has described small-molecular-weight proteins located in vesicles in the cytoplasm of *Saccharomyces cerevisiae* which may regulate cell wall synthetic enzymes. The directing of these activators and inhibitors to specific sites could cause localized cell wall biosynthesis. Alternatively, yeast and mycelial cells may contain different activators and inhibitors.

These results support and greatly extend the work of Dabrowa et al. (8), who reported the presence of one yeast- and two mycelium-specific proteins in a cytoplasmic extract containing 12 proteins, and Syverson et al. (35), who identified yeast- and mycelium-specific cytoplasmic components in *C. albicans* by crossed immunoelectrophoresis. Neither of these groups investigated strain- or temperature-dependent protein changes. These data do not completely eliminate the possibility that cytoplasmic proteins entirely unique to mycelial-phase cells or additional yeast phase-specific proteins are synthesized. Certain cytoplasmic proteins may lack sulfur or may be present in amounts too small to be detected by this gel system. O'Farrell (28) reported that proteins possessing only 1 dpm of ^{14}C or ^{35}S and comprising 10^{-5} to 10^{-4}% of the total protein in the sample should be detectable. The use of PPO incorporation and preflashed film (3, 17) increased the sensitivity of the system by a factor of 40. All gels analyzed in this study were exposed initially for 5 to 6 weeks and the films were developed. Reexposure of the same gels for an additional 10 to 11 weeks did not reveal any additional spots, but small areas of each gel were rendered unintelligible as a result of overexposure.

Through the gel analysis technique developed for this investigation, proteins associated with a particular strain or cultivation temperature were also identified. Moreover, Table 1 and Fig. 2 allow the rapid identification of proteins with any desired combination of characteristics (e.g., a protein present only in strain 4918 and absorbed only by anti-4918-37M immunoglobulin). The gels, however, contain much more information than is readily accessible by this method of analysis. For example, changes in spot intensity were not recorded for the comparison of the reference or pulse-chase gels. Computerized methods of cataloging all the spots on a gel are being developed (18). A practical application of these techniques is in the area of taxonomy. Similarity coefficients, calculated from the number of shared proteins between test organisms, should reflect the degree of taxonomic relatedness. As expected, very few proteins were recovered by normal rabbit serum or staphylococcal protein A alone. The few spots that were removed by normal rabbit (preimmune) immunoglobulin but not specific immunoglobulin are unexplained (e.g., HI'i°). Detailed analysis of the antibody cross-absorption data are presented elsewhere (Manning and Mitchell, in press).

The techniques and results described herein suggest several avenues of future research. Yeast- or mycelial-phase protein could be localized by two-dimensional analysis of subcellular fractions (e.g., cytoplasmic vesicles or nuclei). The membrane-bound protein population of the two forms could be investigated. The yeast- and mycelium-associated proteins could provide an earlier marker of a cellular commitment to morphogenesis than conversion (or reversion) per se, which requires at least 60 min to be morphologically detectable. Such a marker would allow the molecular regulation of dimorphism to be more closely monitored during investigations utilizing effectors of morphogenesis or macromolecular synthesis. For example, Ogletree et al. (29) reported that rifampin and amphotericin B in combination inhibit both germ tube production and budding. Other reports have suggested

that phenethyl alcohol may inhibit yeast to mycelium morphogenesis of *C. albicans* on solid medium (19) and in serum (13). Similarly, morphology-dependent protein markers could facilitate studies of the morphogenic autoregulatory substance which Hazen and Cutler (12) found to be associated with the yeast phase of *C. albicans* (including strain 2252).

Further investigations would be greatly facilitated by the use of an isogenic mutant of *C. albicans* wherein the mutant and the wild type produced the two growth forms under the same environmental conditions.

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