Dimorphism in *Histoplasma capsulatum*: a Model for the Study of Cell Differentiation in Pathogenic Fungi

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INTRODUCTION

Pathogenic fungi such as *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Sporothrix schenckii*, and *Paracoccidioides brasiliensis* have the capacity to grow in either a yeastlike or a mycelial form in response to environmental stimuli. Such stimuli include changes in CO₂ tension, the temperature of incubation, oxidation-reduction (redox) po-
tential, and nutritional factors. The ability to exist in different forms is called dimorphism. This type of cell differentiation in fungi is distinct from processes such as embryogenesis in higher eukaryotic organisms since it is a reversible process and is not an essential part of the life cycle of the organism.

An important feature of morphogenesis in some dimorphic fungi is that induction of one phase leads to the form found in infected tissue. Dimorphic pathogenic fungi have a unique ability to colonize host tissue that is parallel to and may be intimately involved with the developmentally regulated morphological transition.

This review deals primarily with the biochemistry and recent findings on the molecular biology of *H. capsulatum* and related features of the process of differentiation. Other detailed reviews have already been published on the growth requirements (50), antigenic studies and chemistry of the cell wall (127, 140), physiological studies (12, 80, 100), electron microscopic studies (37, 38), and the epidemiology and clinical aspects of histoplasmosis (55, 56, 71, 155, 168).

**THE DISEASE**

Histoplasmosis, a systemic fungal disease, is caused by *H. capsulatum var. capsulatum*. It is worldwide in occurrence and represents the most common respiratory mycotic infection affecting humans and other animals (155). The infection has a particularly high prevalence in temperate, subtropical, and tropical zones such as the Mississippi and Ohio Valleys in the United States (1, 33, 155) and in portions of South and Central America (33, 94). It is also widely diffused in the Mediterranean area (155), Asia (1), Australia (75, 136), and Africa (97, 155). In addition, a distinct clinical form of the disease called histoplasmosis duboisii, caused by *H. capsulatum var. duboisii* (128), occurs in Africa.

While clinical histoplasmosis covers a wide range of symptoms, there are only a few clues to the strain specificity and pathophysiology of the infection. Though the environmental and genetic factors that result in a "successful" infection are only partially known, progressive disseminated histoplasmosis has increasingly become an important opportunistic infection as a result of deficiency of host defense mechanisms responsible for the intracellular killing of fungi (169). Recently, basic studies on *H. capsulatum* have received a greater focus of attention, due in part to the increasing prevalence of these infections in immunosuppressed or otherwise debilitated patients, particularly with the advent of the acquired immune deficiency syndrome. Histoplasmosis in patients with acquired immune deficiency syndrome occurs commonly in areas where histoplasmosis is endemic (71) but has become an increasing concern in nonendemic areas due to the mobility of the population (66).

**THE ORGANISM**

From a biochemical and molecular point of view, *H. capsulatum* is the most extensively studied of the dimorphic fungi, with a parasitic phase consisting of yeast cells and a saprobic mycelial phase. Yeast cells are oval (1 to 3 μm in diameter; Fig. 1), localize in macrophages and reticuloendothelial cells (Fig. 2), and grow in culture at 37°C. In culture, yeast cells reproduce by budding, with the presence of a single nucleus in unbudded cells (31). The doubling time of yeast phase cells at 37°C is about 6 h in glucose-yeast extract supplemented with spent medium (B. Maresca, unpublished data) and 12 to >30 h in a synthetic medium supplemented with cysteine or serine or both (7, 78, 159, 170). Hyphae measure approximately 1.2 to 1.5 μm in diameter (Fig. 3) and grow more slowly, with a mass doubling time in liquid culture at 25°C of no more than once over 24 h. The hyphae produce, in appropriate conditions, two types of conidia: macroconidia (23, 57) and microconidia (32, 63). Microconidia, probably due to their small dimension (<5 μm in diameter), are the preferential infectious form of *H. capsulatum*. Microconidia, which have a large number of external tubercules, can germinate to form yeast cells by polar or nonpolar budding at 37°C (36, 40, 130).

Yeast cells are the only form of the organism observed in infected tissue. Very little has been reported about the transition of microconidia to yeast cells at 37°C. In the
transformation of microconidia to mycelium, 48 h after the shift in temperature, they develop a structure similar to buds, with a wall 75 nm thick in appropriate sporulation medium (88); after about 72 h, the budlike structure elongates into short septate hyphae (40).

A sexual phase of *H. capsulatum* has been described by Kwon-Chung (87), is classified as an ascomycete, and was originally designated as Emmonsia capsulata (subsequently renamed Ajellomyces capsulatum [105]). *A. capsulatum* is heterothallic; when (+) and (−) mating types are seeded onto sporulating medium, cleistothecia (macroscopic fruiting bodies containing asci) are produced (88). It is interesting to note that patients with histoplasmosis preferentially (7:1) carry the (−) mating-type form in their tissues (89). Upon microscopic examination, mycelia of *H. capsulatum* (particularly on primary isolation) show a wide range of morphological characteristics. Berliner showed that isolates, either from animals or from soil, produce a brown colony (B), which in appropriate conditions upon subculture gives rise to B and A, white colonies (where A is albino [4, 5]). They are both phenotypically stable and can be maintained under laboratory conditions. The A type grows rapidly, lacks any apparent pigment, and, after repeated passages, no longer produces spores. In a mixed culture with the B type, the A type will eventually predominate. The B type produces a brown pigment and shows a large number of macroconidia and few microconidia. Daniel et al. showed that the yeast phase of the B form is more pathogenic than the A form when injected into rabbits (24). Later, Tewari and Berkhourt did a similar study in mice, with similar results (163). So far, there is no explanation for the greater virulence of the B form or for the gradual conversion of the B colonies into the A form.

**FIG. 2.** Yeast cells within monocytes.

**Strains and Virulence**

The lack of suitable genetic markers and biochemical and serological tests has hampered the development of a reliable classification scheme to classify and determine interrelatedness of different isolates of *H. capsulatum*. Vincent et al. studied 23 isolates from human and animal sources from diverse geographical origins (North America and Africa) (167). Based on restriction fragment patterns of mitochondrial deoxyribonucleic acid (mitDNA) and ribosomal DNA (rDNA), the strains fell into three distinct classes (Table 1) (167). Each restriction endonuclease produced a characteristic pattern of the mitDNA for each class. The molecular weights of the mitDNAs of the strains in each class were 33,000, 38,000, and 47,000 for classes 1, 2, and 3, respectively. The rDNA repeating units of classes 1, 2, and 3 were also characteristic: 6,900, 7,300, and 8,400 molecular weights, respectively. Of the 23 strains analyzed, 16 belong to class 2, as defined by mitDNA and rDNA restriction patterns, and included both isolates of *H. capsulatum var. dakoisi*. Class 3 was composed of strains isolated in Central and South America. The only member assigned to class 1 was the Downs strain. Recently, Spitzer et al. (E. D. Spitzer, B. A. Lasker, G. S. Travis, G. S. Kobayashi, and G. Medoff, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, p. 397; E. D. Spitzer, personal communication) initiated a study of restriction fragment length polymorphism of mitDNA and rDNA of soil isolates to characterize the degree of genetic diversity among strains that are not associated with the disease in comparison to clinical patterns. Of nine isolates (all of North American origin), eight were identical to class 2, while the remaining one was distinct from the
three previously described mitDNA classes and has been assigned to a new class, class 4. It would be interesting to determine the level of virulence of the new class 4 isolate to see whether there is any correlation between DNA polymorphism and pathogenicity.

More recently, three clinical isolates of *H. capsulatum* have been found to have ribosomal and mitochondrial restriction fragment length polymorphisms identical to those of Downs and have been included in class 1 (Spitzer, personal communication). These strains have additional properties similar to those of the Downs strain, including lower infectivity in mice and greater temperature sensitivity than more virulent strains.

### TABLE 1. Source and class of isolates of *H. capsulatum*

<table>
<thead>
<tr>
<th>Class</th>
<th>Isolate (source)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Downs (human)</td>
<td>North America (55)</td>
</tr>
<tr>
<td>2</td>
<td>2310 (human)</td>
<td>North America (A. Body, Lexington, Ky.)</td>
</tr>
<tr>
<td>2</td>
<td>6617 (human)</td>
<td>North America (142)</td>
</tr>
<tr>
<td>2</td>
<td>6622 (cat)</td>
<td>North America (142)</td>
</tr>
<tr>
<td>2</td>
<td>6623 (opossum)</td>
<td>North America (142)</td>
</tr>
<tr>
<td>2</td>
<td>6624 (human)</td>
<td>North America (142)</td>
</tr>
<tr>
<td>2</td>
<td>6628 (human)</td>
<td>North America (142)</td>
</tr>
<tr>
<td>2</td>
<td>UCLA 505 (human)</td>
<td>North America (D. H. Howard, UCLA, Los Angeles, Calif.)</td>
</tr>
<tr>
<td>2</td>
<td>1073 (human)</td>
<td>North America (142)</td>
</tr>
<tr>
<td>2</td>
<td>District (human)</td>
<td>North America (L. Pine, CDC, Atlanta, Ga.)</td>
</tr>
<tr>
<td>2</td>
<td>Grand Island (human)</td>
<td>North America (L. Pine, CDC, Atlanta, Ga.)</td>
</tr>
<tr>
<td>2</td>
<td>2k (human)</td>
<td>North America (142)</td>
</tr>
<tr>
<td>2</td>
<td>G217A (human)</td>
<td>North America (5)</td>
</tr>
<tr>
<td>2</td>
<td>G217B (human)</td>
<td>North America (5)</td>
</tr>
<tr>
<td>2</td>
<td>G222B (human)</td>
<td>North America (5)</td>
</tr>
<tr>
<td>2</td>
<td>Duboisii 2100 (human)</td>
<td>Africa (L. Ajiello, CDC, Atlanta, Ga.)</td>
</tr>
<tr>
<td>2</td>
<td>Duboisii 2591 (human)</td>
<td>Africa (L. Ajiello, CDC, Atlanta, Ga.)</td>
</tr>
<tr>
<td>3</td>
<td>G184A (human)</td>
<td>Central America (4)</td>
</tr>
<tr>
<td>3</td>
<td>G184B (human)</td>
<td>Central America (4)</td>
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<tr>
<td>3</td>
<td>G186A (human)</td>
<td>Central America (4)</td>
</tr>
<tr>
<td>3</td>
<td>G186B (human)</td>
<td>Central America (4)</td>
</tr>
<tr>
<td>3</td>
<td>H2 (human)</td>
<td>South America (A. Restrepo, Medelin, Colombia)</td>
</tr>
<tr>
<td>3</td>
<td>H2 (human)</td>
<td>South America (A. Restrepo, Medelin, Colombia)</td>
</tr>
</tbody>
</table>

* Table was modified and reprinted with permission from the publisher of reference 167. CDC, Centers for Disease Control.

### Chromosomes


Assignment of specific DNA probes to individual bands has also been accomplished by Steele et al. with blots of gels in which five bands are resolved (with this technique, two bands represent doublets) (Steele et al., Abstr. Annu. Meet. Am. Soc. Microbiology 1988) in an effort to begin to determine linkage groups in this fungus. Cloned *H. capsulatum* α-tubulin and actin genes map to the fastest-migrating chromosomal band. Probes from *H. capsulatum* β-tubulin and rDNA map to other bands. Random recombinant plasmids containing nonidentified *H. capsulatum* DNA sequences have also been used to identify each of the other large DNA bands resolved by field inversion gel electrophoresis. The use of these random clones in blot hybridization of field inversion gels has shown that each of these strains has a unique banding pattern. For example, G184B and G186B.
which have identical mitDNA and rDNA restriction patterns, are quite different in probe assignments to their chromosomes.

**Morphology**

Electron microscopy has provided information about both the ultrastructure of fungal cells and changes during dimorphism (38). From a morphological point of view, however, cytological features do not distinguish pathogenic dimorphic fungi from other nondimorphic pathogenic (37) or nonpathogenic (15) fungi.

**Yeasts.** Generally, fungal yeasts have thin cell walls (Fig. 4); in the case of the pathogenic fungus S. schenckii, the outermost wall surface is covered with a layer of microfibrils (45). Garrison and Arnold have shown that these wall microfibrils may associate with certain enzymatic activities such as acid phosphatase (39). Most yeast cells of dimorphic fungi reproduce by multipolar budding; the bud cell wall arises from an innermost layer of the parental cell wall. Electron microscopy techniques have revealed a variety of organelles and inclusions in the cytoplasm of the yeast cell. Yeast cells of B. dermatitidis and P. brasiliensis are multinucleate, whereas yeast cells of H. capsulatum are uninucleate. As in all eucaryotic cells, the endoplasmic reticulum is present throughout the cytoplasm and may communicate with the nuclear membrane; ribosomes are present and found in association with the endoplasmic reticulum, and mitochondria are usually elongate. Vacuoles are present in old cells and seem to possess lysosome-like functions, since some lysosomal marker enzymes have been demonstrated cytchemically to be localized within H. capsulatum cells (46). Microbodies, such as glyoxysones and peroxysomes, have not been described in dimorphic pathogenic fungi.

**Hyphae.** The distribution of organelles and inclusions within hyphal cytoplasm is generally quite similar to that seen in yeast cells (Fig. 5). The mycelial phase of H. capsulatum consists of seolate hyphal elements. Hyphal units of H. capsulatum are thin (1.25 to 2.0 μm in diameter) and have a bilaminar wall 20 nm thick. Tip cells are either uninucleate. During mitosis, nuclei undergo synchronous division in uninucleate cells (80). Multivesicular bodies that may aggregate are present in the subapical region. Golgi-like cisternae are present in large number in H. capsulatum hyphae. Septa possess a narrow central pore (100 nm in diameter) that may become occluded with a Woronin body. Woronin bodies have been described as a class of round to elongated intracytoplasmic organelles usually associated with septal pores of ascomycetous fungi (37). They are believed to act as plugs in pores that regulate

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**FIG. 4.** Composite representation of a yeast cell showing microfibrillar material (MFM) at the cell wall (CW) surface, a bud scar (bs), a bud cell (bc) and the origin of its wall from an inner layer (IL) of the parent wall, lomasome (L), plasma membrane (pm) and its invagination (i). nucleus (Nu), nucleolus (NC), nuclear membrane (nm), nuclear pore (np), endoplasmic reticulum (ER), ribosomes (R), lipid body (LB), membrane system (ms), multivesicular body (mVB), mitochondrion (Mi) and its cristae (c), glycogen-like material (G), vacuole (V), and fungal lysosome-like equivalent (FLe) containing an electron-opaque body (eob). A highly structured Golgi apparatus (ga) does not occur. Microbodies (mb), microfilaments (mf), and microtubules (mt) likely occur, but have yet to be described in detail. Reprinted from reference 38 with permission of Plenum Publishing Corp.

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**FIG. 5.** Composite representation of an ascomycetous hypha showing wedge-shaped septal plates (S), septal pore (SP) and associated Woronin bodies (WB/wB), cell wall (CW), plasma membrane (PM), lomasome (L), glycogen-like aggregates (G), lipid body (LB), endoplasmic reticulum (ER), mitochondrion (Mi), membrane system (MS), and nucleus (Nu) and nucleolus (NC). Reprinted from reference 38 with permission of Plenum Publishing Corp.
cytoplasmic flow between adjacent hyphal cells. So far, no enzymatic function has been associated with these structures. Hyphal and yeast cells of *B. dermatitidis* possess structures similar to those described for *H. capsulatum*. In addition, an extensive lamellar intracytoplasmic membrane system has been described whose function is presently unknown (27, 44).

**Yeast-to-hyalp cell transition.** The yeast-to-hyalp cell transition has been studied by electron microscopy and is well documented for *P. brasiliensis* (16, 73), *B. dermatitidis* and *H. capsulatum* (38, 44, 73), and *S. schenckii* (38, 43, 93). Yeast cells differentiate to mycelia in all of these fungi at an incubation temperature of 22 to 25°C. Yeast cultures are not synchronized, so that morphological changes do not occur at the same time in all cells, but the transition begins with the formation of a budlike structure. This structure contains large quantities of storage material. Occasionally, with the formation of a budlike structure. This structure can be seen (93). Very similar behavior has been reported for *H. capsulatum* (27, 154; G. Garrison et al. have investigated the morphogenetic process from mycelium to yeast cell of *P. brasiliensis* with a light microscope. As observed in *H. capsulatum* and *B. dermatitidis*, most hyphal walls form cell walls, and only after 18 h were any cell modifications visible. Observed by electron microscopy, hyphae seem to increase their diameter; the outer wall layers break and the inner layers are in contact with the cytoplasm (38). As the transition proceeds, the thickness of the cell wall increases along with the fragmentation of the outer layers. As the space between the cell walls increases, the cell tends to become rounded and separate.

Howard has described the morphological events of the hyphal to yeast transition in *S. schenckii* (62). He described two different processes by which yeast cells developed from hyphal: (i) formation of buds at the tips of hyphal branches and (ii) formation of oidiad-like structures, followed by the fragmentation of the hyphal that leads to yeast cells. Garrison et al. have confirmed this description at the ultrastructural level (41).

**Nutritional Requirements**

Most of the early studies on the growth requirements of the mycelial and yeast phases of *H. capsulatum* have been reviewed by Gilardi (50). In recent years, studies have focused mainly on the role of cysteine in the yeast phase and the mycelium to yeast transition. Also, several groups have tried to define a synthetic medium that allows the incorporation of labeled precursors to study ribonucleic acid (RNA) and protein synthesis. In contrast, little has been done recently on the nutritional requirements of the other dimorphic pathogens.

**Vitamins.** Salvin was the first to define nutritional differences of yeasts cells and mycelia of *H. capsulatum* (147). He showed that biotin was a specific requirement for the yeast phase of some strains, but not for the mycelial phase. Other studies on vitamin requirements have given variable results: the mycelial form has been found to require no vitamins at all (125, 148) or to require thiamine (153), pantothene (142), or niacin (153). The yeast form has been found to require thiamine and thiotic acid (125) and biotin (125, 148). The discrepancy among these data is very likely due to the lack of a defined medium in which to grow cells and possibly to a high variability in the growth requirements of isolates of different origins.

**Amino acids.** Studies on amino acid requirements have had similar complex results (142). Salvin showed that the mycelial phase of *H. capsulatum* grows with ammonium as the sole source of nitrogen and requires sulphydryl (—SH) groups in the form of cysteine (148). His most important finding was that the addition of cysteine or cystine to a glucose salts medium was necessary for the maintenance of the yeast phase (148). That observation has been amply confirmed (123, 124, 154; G. H. Scherr, Bacteriol. Proc., p. 85, 1956). Pine (123) has found that, in addition to cysteine, aspartic and glutamic acids are stimulatory for growth. He also found that glutathione can be substituted for —SH requirements but not for cysteine. This was the first suggestion that cysteine was not required for —SH groups alone. A second role for cysteine during the phase transition, distinct from a growth requirement for the yeast phase, has also been reported (see subsection, "Sulfur Metabolism").

**Factors Influencing Dimorphism**

Dimorphism in *H. capsulatum* is directly mediated by temperature changes (125, 129, 147, 154; Scherr, Bacteriol.
Proc., p. 85, 1956), once other conditions, such as the presence of vitamins (125, 148, 149) and SH groups (123, 124, 148; Scherr, Bacteriol. Proc., p. 85, 1956), are satisfied. The optimal temperature for the maintenance of the yeast phase is 37°C; for mycelia, it is 25°C (125, 147, 154; Scherr, Bacteriol. Proc., p. 85, 1956). The mycelium to yeast conversion occurs above 30°C, with an optimum at 37°C (154). Recently, it has been suggested that the optimum temperature for mycelium to yeast transition in a given strain is directly dependent on genetic determinants that are strictly correlated with temperature (18, 111). However, it is clear that SH-containing compounds must be present in the culture medium to initiate the mycelium to yeast transition. Scherr speculated that at least two factors would influence the mycelium to yeast phase transition: (i) SH-containing compounds might represent a nutritional requirement in the yeast phase to compensate for an inability of the yeast phase to reduce sulfur-containing compounds at 37°C; or (ii) —SH groups might regulate in some manner the redox potential of the growth medium necessary for yeast development (154). Rippon showed that conversion of mycelium to yeast could be obtained without adding cysteine at any temperature of incubation, as long as the redox potential of the medium had been lowered to +46 mV; furthermore, the mycelial form could be maintained at +380 mV (138, 140). Rippon obtained these results by growing H. capsulatum in a chamber in which the environment was artificially monitored at a desired redox potential. Unfortunately, this work is difficult to interpret since a rich medium (brain heart infusion) rather than a chemically defined one was utilized, and a variety of sulfur-containing compounds were undoubtedly available.

The analysis was furthered by McVeigh and Houston (108), who pointed out that, although the transition could be induced at reduced oxygen levels and in the absence of cysteine, both were needed for the conversion of mycelia to yeast cells and for maintenance of the yeast phase: cysteine could not be replaced by glutathione in either case. These findings were supported by findings which described three distinct stages during mycelium to yeast transition (102). During the early period of shift-up in temperature in the Downs strain of H. capsulatum, cell metabolism slowed drastically and cells entered a dormant phase during which there was no measurable oxygen consumption. Cysteine was also implicated in mitochondrial respiratory pathways (see the section on physiology and references 42, 82, 101–103, 144 and 160). The requirement for cysteine to complete the mycelium to yeast transition was clearly separated from the requirement of yeast cells for cysteine.

Yeast cultures of H. capsulatum spontaneously transform to mycelia when they reach stationary phase at 37°C, probably due to the exhaustion of some specific compound(s). In similar growth media, mycelia do not transform spontaneously to the yeast phase at low temperatures. Furthermore, it has been shown that a number of substances can influence the yeast to mycelium conversion at 37°C (see subsection, “Other Factors Inducing Morphogenesis”) (103, 143). This would imply that, while H. capsulatum can grow as a mycelium regardless of the temperature of incubation, the mycelium to yeast transition and maintenance of the yeast phase morphology require 37°C. This supports the notion that ‘mycelium-specific genes’ may be expressed at any temperature, while ‘yeast-specific and transitional specific genes’ are regulated and can be expressed only at high temperature in the presence of —SH groups in the medium.

The yeast phase of S. schenckii grows in rich media containing glucose as carbon source. Thiamine is an essential growth factor for the yeast phase (50, 139). Most of the studies on amino acid requirements have been done with media containing a complex organic nitrogen source, such as peptone or tryptone. It has been reported that the yeast phase again requires cysteine (165), though it may be necessary as a substrate able to lower the redox potential of the medium rather than a true amino acid requirement. Yeast cells develop in 5% CO₂ tension in a medium containing ammonium sulfate and asparagine at 37°C; in the presence of N₂ alone, in contrast, mycelia develop (141). Further, in a chemically defined medium, yeast cells can be maintained at temperatures as low as 25°C (115). This implies that dimorphism in S. schenckii is controlled by temperature, nutritional factors, and CO₂ concentration. There is not yet any mechanistic study of how these factors interact or control gene expression.

In liquid culture, the phase transition of P. brasiliensis is totally temperature dependent, with mycelia at room temperature and yeast cells at 37°C (51, 162). In glucose-deficient media, P. brasiliensis produces arthroconidia and basidioconidia (131). In vitro on complex media at 37°C and in tissues, P. brasiliensis grows as a multinucleate yeast up to 30 μm in diameter (38, 46) which multiplies by polar and nonpolar budding. Several authors (121, 134, 137) have reported that P. brasiliensis requires a sulfur-containing amino acid for maintenance of the yeast phase.

B. dermatitidis exhibits a yeast–mycelium dimorphism primarily regulated by temperature. Halliday and McCoy, in a study of six different strains, determined that B. dermatitidis requires biotin as growth factor (59). However, Gilardi and Laffler reported that B. dermatitidis requires neither amino acids nor vitamins for growth (51).

Most of the studies on nutrition have contributed little to the understanding of morphogenesis in dimorphic fungi since they were conducted with poorly defined isolates, different media, different growth conditions, etc. Nevertheless, they are an initial approach to the study of the physiology of these fungi, and, in particular, requirements for special —SH or redox conditions recur in studies on dimorphic fungi. For all of them, the use of standard laboratory strains and chemically defined media would be necessary starting points for biochemical and genetic analyses.

**PHASE TRANSITION**

Phase transitions of H. capsulatum can be easily accomplished, reversibly, under laboratory conditions by shifting the temperature of incubation from 25 and 37°C. This implies that dimorphic fungi do not become committed to a specific cellular stage and that genes must be regulated by a combination of temperature and any other environmental factors that turn them on and off in an entirely reversible manner. Therefore, these systems differ from all of those in which organisms undergo a “developmental process” which is generally irreversible. Detailed analysis, at the genetic and functional levels, is thus of especially great importance in understanding the relationship of this pathogen with humans.

**Sulfur Metabolism**

Early studies with H. capsulatum showed that the “natural signal,” a shift in temperature, cannot itself be indispensable to induction of the mycelium to yeast phase transition (138, 148, 154). Rather, temperature seems to affect the redox state of the —SH groups or the “general”
redox potential in cells or both, which would then determine the phase of the organism. The possible role of sulfur-containing compounds, such as cysteine and cystine, has induced a number of investigators to study the uptake, biosynthesis, and metabolism of these compounds in an attempt to ascertain their possible role in determining the phase of the organism.

**Cystine and cysteine uptake.** Garrison et al. (42) and Gilbert and Howard (52, 53) have shown that transport of cystine into the yeast phase of *H. capsulatum* is an adenosine 5′-triphosphate (ATP)-dependent process. Gilbert and Howard have shown that the permease system is specific for cystine and is not inhibited by other amino acids (53). Garrison et al. have further demonstrated that cysteine and cystine stimulate cellular respiration of the yeast, but not the mycelial, phase of *H. capsulatum* (42). It was shown later that yeast cells and mycelia take up of cysteine equally well, at an uptake rate of 3 μmol/min per mg of protein at 37°C (98). At 25°C, the rate of uptake by each phase was approximatively halved. Variants blocked in the metabolism of cystine were also selected by using the toxic analog selenocystine (98). Two of these mutants, sec-1′ and sec-2′, independently derived, were shown to be impaired in cystine uptake (cystine uptake was reduced to ca. 10% of the value of control cells). Despite the defects in cystine uptake, both mutants were able to undergo mycelium to yeast and yeast to mycelium transition at the same rate as the parental strains. The uptake experiments and the studies of the variants, sec-1′ and sec-2′, supported the idea that cystine uptake is not a requirement for maintenance of the yeast phase.

Using the same genetic approach, Jacobson and Harrell have studied the role of cysteine in the growth of the yeast phase of *H. capsulatum* and during mycelium to yeast transition (67). They analyzed mutants previously generated with genetic blocks in the metabolism of cystine by selection of colonies resistant to the toxic analog selenocystine (up to a concentration of 3 mM [98]). Using the Downs strain, they isolated 20 additional mutants deficient for cystine uptake. For example, some of the mutants were capable of only 5% of the uptake activity of the Downs parental strain. All members of this class of mutants, like those selected previously, had normal reduced nicotinamide adenine dinucleotide-dependent cystine reductase activity. Further, while all mutants could be converted to the yeast phase (and back to mycelia), the mycelial phase of the mutants was always strongly inhibited by selenocystine. They concluded, too, that cystine uptake was not a critical step in mycelium to yeast phase transition in *H. capsulatum*. Jacobson and Harrell have investigated further the role of cysteine by isolating, from the Downs strain, a prototrophic yeast mutant (H-35) that could grow and transform to the yeast phase in a minimal medium containing glucose, biotin, tartrate, and inorganic salts but no cysteine (68). Incidentally, they also described the production of cysteine-requiring revertants of the prototrophic strain H-35 (69). According to Jacobson and Harrell (69), the isolation of a prototrophic mutant able to grow and transform to the yeast phase implied that cysteine was not a requirement for *H. capsulatum*. It is also possible that if a mutant is able to perform a function not present in a parental strain, the specific function is indeed required in the parental strain. However, a correct interpretation of the role of cysteine during phase transition requires more specific information, especially when dealing with mutants. In fact, isolation of ultraviolet light- or nitrosoguanidine-generated mutants always raises the possibility of producing pleiotropic strains whose behavior may lead to misleading conclu-

sions, as has been shown in the study of mutants generated at various stages of morphogenesis during spore formation in *Bacillus subtilis* (122).

Howard et al. have isolated a mutant (NG-2) defective in the ability of its blastospores to germinate (64). They showed that the nutritional requirement for cysteine and the expression of sulfite reductase are temperature dependent and not yeast phase specific (on the contrary, the kinetics of cysteine uptake depends on both temperature and phase of the fungus).

Further genetic studies should be useful in defining the role that sulfur compounds play in the mycelium to yeast transition and in maintenance of the yeast morphology. These studies may prove to be difficult from a genetic point of view since they will depend on a careful characterization of the role that cysteine plays in controlling gene expression.

**Cysteine and glutathione reductase.** Cysteine reductase (EC 1.6.4.1.), a reduced nicotinamide adenine dinucleotide-dependent enzyme, has been shown to be associated with the plasma membrane fraction of the yeast phase of the fungus (81, 98) and to be induced during the first 4 h of the transition of mycelium to the yeast phase at 37°C (98). In contrast, glutathione reductase activity (EC 1.6.4.2.; a reduced nicotinamide adenine dinucleotide phosphate-dependent cytoplasmic enzyme) was shown to be in both yeast cells and mycelia (98). These authors concluded that cysteine reductase is a yeast-specific enzyme and may play a role in mycelium to yeast phase transition by producing, at the membrane level, enough reducing equivalent in the form of cysteine to trigger transition.

**Sulfite reductase.** The enzyme sulfite reductase has also been suggested to play a role in controlling phase transition in *H. capsulatum* (7). This enzyme, which reduces sulfite to sulfide (SO₄²⁻ → S⁰), is repressed in yeast cells at 37°C and thus cannot synthesize cysteine. Later, Stetler and Boguslawski extended this study to several strains of *H. capsulatum* in addition to Downs and concluded that the yeast cysteine requirement was due to the absence of an active form of sulfite reductase, an enzyme usually needed for cysteine biosynthesis (159).

**Protein P.** Stetler et al. characterized a yeast-phase-specific protein, termed protein P, which was induced in cells derepressed for sulfite reductase (160). Such protein, which can account for as much as 40% of total protein in cell extract, is subject (as is sulfite reductase) to cysteine repression. Protein P was shown to be synthesized in growing cells de novo and to accumulate in stationary-phase cells. The molecular weight of this protein in sodium dodecyl sulfate gel is ca. 7,600. It has reducing properties, as shown by its ability to reduce cytochrome c and Nitro Blue Tetraxazolium. Even though cells synthesize large quantities of this protein, implying an important role, no function has been assigned to this polypeptide.

**Physiology: Role of Cysteine in Regulating Morphogenesis and Mitochondrial Activity.**

Cell respiration. The role of cysteine during morphogenesis has been analyzed by studying the effect of this amino acid on respiration of both phases of *H. capsulatum* and during the transitions (101). As in *Neurospora crassa* and other fungi (91, 92), respiration in *H. capsulatum* proceeds through a branched electron transport system consisting of two terminal oxidase pathways: one, the cytochrome system, is inhibited by cyanide and antimycin; the other, an unidentified alternate oxidase, insensitive to those inhibi-
Cysteine oxidase. Cysteine oxidase (or cysteine dioxygenase: l-cysteine:oxygen oxidoreductase, EC 1.13.11.20) has been purified from the cytosolic fraction of yeast phase cells in an attempt to understand its possible role in the transition (82). This enzyme is probably the only one ever isolated from a fungus. In *H. capsulatum*, the product of oxidation, as in other systems (19, 145, 158, 171), was cysteine sulfinic acid, identified by thin-layer chromatography and mass spectroscopy. The enzyme isolated from the fungus is an iron-containing dioxygenase (as established by the increase in activity after preincubation with Fe(II) with a molecular weight of 10,500 (±1,500), in contrast to the rat liver enzyme, which has a molecular weight of 22,500. The protein activity (and probably the protein itself) is absent in the mycelial phase and appears within 3 to 4 days after the temperature is brought to 37°C (102). Cysteine sulfinic acid, the product of oxidation, is considered a key intermediate of cysteine metabolism; therefore, it has been suggested that this enzyme may regulate the level of intracellular free cysteine or provide a metabolic product of the oxidation of cysteine important for the mycelium to yeast transition (82).

Mitochondrial activity. Three distinct stages have been distinguished in the transition following the temperature shift from 25 to 37°C in the Downs strain of *H. capsulatum* (102). Stage 1, immediately after the temperature shift and encompassing the first 24 to 40 h, was characterized by a progressive decrease in cellular respiration rate of mycelia, with a parallel two- to fivefold decrease in intracellular cysteine concentration and other amino acids (102). The depletion of free amino acid pools may reflect a decreased rate of biosynthesis or transport due to depletion of ATP as supported by data on decrease of RNA synthesis (20) and uncoupling of oxidative phosphorylation (90, 111). In stage 2, the cells showed no respiration and were essentially dormant. After 4 to 6 days, cells entered stage 3, which is characterized by an increase in the concentration of intracellular cysteine and other amino acids and then by the return of respiration to the level characteristic of the yeast phase. The early morphological changes were observed microscopically by these authors at the end of stage 2 to early stage 3. It was also reported that the addition of exogenous cysteine or other reducing agents shortened the dormant stage, accelerating the mycelium to yeast conversion. Furthermore, mitochondria isolated from dormant-state cells (stage 2) did not respire with any substrate tested (reduced nicotinamide adenine dinucleotide, succinate, malate, etc.). However, these substrates were oxidized when mitochondria were first treated with 1.6 mM cysteine or 2 mM β-mercaptoethanol. Whole-cell respiration was also shown to be activated and restored by addition of exogenous —SH groups. Remarkably, cysteine acted by affecting mitochondrial respiration. Therefore, in the absence of cysteine, cells could only progress up to stage 2 of transition: cysteine or other —SH-containing compounds are required to complete transition by permitting the cells to enter stage 3 (102). The requirement for cysteine for completion of transition is strictly dependent on the degree of thermosensitivity of the strain (see below). The role of cysteine, as it was suggested in this study, is also in agreement with the former observations by McVeigh and Houston (108). They had, in fact, shown that the induction of the mycelial to yeast phase could occur in the absence of reduced —SH-containing compounds (stage 1) and molecular oxygen (stage 2), but that cysteine and oxygen were required later to complete the transition and support yeast growth (stage 3). A novel —SH-induced "shunt pathway" was described in a detailed study by Sacco et al., who found that cysteine has a second effect on oxygen consumption that is separate from the function of cysteine oxidase (144). They showed that, during stage 2 of the mycelium to yeast transition of the Downs strain, the decrease in respiration was correlated with a decrease in the concentration of electron transport components. By analyzing 77 K difference spectra of mitochondria isolated from cells at different time points following the shift from 25 to 37°C, they showed a five- to sixfold decrease in the contents of electron transport components within 24 h. At this time point, the predominant component was a type b cytochrome (a peak at 558 nm). This component was reduced by diithionite, but not by succinate or succinate plus cysteine, suggesting that it was non-functional or that a block, prior to cytochrome b, was present. After 7 days, cytochromes started to increase again and returned to normal within 13 days. The changes in cytochrome content could be correlated with the decrease in cellular respiration and, more important, to the response of cellular respiration to cysteine. Both the cytochrome and the SHAM alternate pathways decreased in parallel. At the 24-point, cysteine-stimulated respiration proceeds through the cytochrome and the alternate pathways (stage 2). In stage 3, cysteine stimulated respiration through the cytosolic cysteine oxidase. These findings allowed Sacco et al. to conclude that cysteine in stage 2 acts by partially reactivating the mitochondrial electron transport components. Further, evidence that cysteine-restored respiration in stage 2 is resistant to 2.5 μg of antimycin per ml while yeast cells are completely blocked by 0.1 μg/ml and mycelia are 50% inhibited by 0.2 μg/ml [144]) suggested the hypothesis that this respiration utilizes a shunt pathway around the antimycin block. To confirm these data, the authors demonstrated that the concentration of cysteine required to overcome the antimycin block in isolated mitochondria (or cells) is identical to that required to restore respiration in stage 2 mitochondria (or cells) in the absence of antimycin. They have postulated a model of the most likely pathways of electron flow and the probable cysteine shunt pathway operative in *H. capsulatum* (Fig. 6). Since cysteine, in the absence of an appropriate respiratory substrate, does not support oxygen consumption in stage 2 mitochondria, they suggested that cysteine itself does not function as an electron carrier, but rather would cause some alteration on either side of the
antimycin block. It is also interesting to note that they have also demonstrated that the cysteine-induced shunt pathways can be induced in both yeast and mycelial mitochondria, but are constitutive only in mycelial cells (probably due to the high intracellular level of cysteine or other —SH-containing compounds). Therefore, it has been pointed out that the shunt pathways in vivo can be correlated with measured levels of intracellular cysteine (102, 144). Thus far, this has been the only report in the literature of —SH-induced shunt pathways, even though cysteine-stimulated respiration has been described in tumor cell lines (99). The authors have speculated that the dependence of the yeast phase on cysteine may reflect an adaptation to a very specific environment. In fact, yeast cells are found in macrophages in which the intracellular environment has high reducing capacity as compared with that encountered by mycelia, which live commonly in soil enriched by bird excreta, probably rich in oxidized compounds. Therefore, they concluded that the dependence on —SH-induced shunt pathways to progress beyond stage 2 may provide a biological control point, which would assure that adequate levels of cysteine are present for transition to proceed.

These studies, far from having explained completely the role of cysteine and —SH groups, give strong support to the concept that cysteine has at least two functions in _H. capsulatum_: (i) cysteine is required to complete mycelial to yeast transition in stage 2, and —SH groups are crucial for this function; (ii) later in transition cysteine acts as a nutrient for yeast cells and may also provide, through cysteine oxidase, a product required for the yeast morphology.

**Heat Shock and Morphogenesis**

Temperature changes and a variety of other stimuli coordinate induce in all organisms vigorous transcription of specific genes (e.g., heat shock genes). The transduction of temperature changes to effect differential gene expression is a central problem in regulating heat shock genes: in dimorphic organisms this problem has important consequences. In fact, temperature works not only as a signal for adaptation (induction of heat shock phenomenon), but also by triggering the phase transition.

**Uncoupling of oxidative phosphorylation.** Since the mycelial to yeast transition is induced by a sudden change in temperature to 37°C, Lambowitz et al. suggested that the early events of the mycelium to yeast transition in _H. capsulatum_ was a “heat shock response” which was followed by cell adaptation to the higher temperature (90). Lambowitz et al. postulated that the triggering event for the biochemical and morphological changes that take place immediately after the temperature shift was a rapid decline in intracellular ATP levels that follows the uncoupling of oxidative phosphorylation. They showed that the intracellular ATP concentration in mycelia shifted to 37°C undergoes a rapid decline: in 5 min it had fallen below the limit of detection, while in yeast cells it was not affected by temperature shift in the reverse transition.

These results led to the novel idea that morphogenesis (or at least the regulation of adaptation to 37°C) in pathogenic fungi (and, in general, in other diphasic organisms) may simply be a by-product of the heat shock response (90).

**Pathogenicity and temperature sensitivity.** A more detailed study on coupling of oxidative phosphorylation during morphogenesis was carried out on two strains of _H. capsulatum_, G184A and G222B, more virulent than the Downs strains (111). These studies showed that the mycelium to yeast transitions induced by a temperature shift from 25 to 37°C are similar to that in the Downs strain, but the changes are less extreme in the more virulent strains. Respiration was only partially uncoupled from oxidative phosphorylation, the fall in ATP levels was only transitory (stage 1), and respiration rates decreased to a much lesser extent (stage 2) than in the Downs strain. Finally, cells recovered to yeast phase (stage 3) in a shorter period of time. These results also showed that the major difference between the more virulent strains and Downs is that the former are more thermotolerant. In fact, the morphological transition of the Downs strain can be made to resemble those of the more virulent strains by shifting it to a lower temperature (34°C). At the same time, the transitions of the more virulent strains can be made to resemble that of the Downs strain by shifting them to higher temperatures (40 to 43°C, depending on the strain used). In all cases, the uncoupling of oxidative phosphorylation is an early and primary event. Further, a correlation was found between sensitivity to elevated temperature during mycelium to yeast transition and virulence for mice.

Therefore, a relationship may exist between the extent of temperature shifts that trigger transition and heat shock response. Lambowitz et al. (90) have proposed that heat shock proteins, among others, may contribute to the adaptation to the new temperature in the early hours of morphogenesis. Since the conversion to the yeast phase appears to be required for infection, it would be likely that the rapidity of the transformation to the yeast phase would be important for virulence.

**Heat shock response.** That a heat shock response was induced at early stages of _H. capsulatum_ morphogenesis was...
directly proven by Schear et al. at the protein level (156) and by Caruso et al. at the gene level (18).

Heat shock proteins are induced during the mycelium to yeast transition in three strains of H. capsulatum at between 34 and 40°C (156). Even though the number of the major heat shock proteins, whose synthesis increase after temperature shift-up, was similar, the pattern of synthesis was quite different and depended on the strains tested and on temperature. Heat shock protein synthesis peaked at 34°C in the Downs strain, whereas in G184A and G222B it was highest at 37°C. The Downs strain might be more sensitive to temperature above 34°C than the other two strains because of the differences in heat shock protein synthesis. Alternatively, the pattern of heat shock protein synthesis in Downs would reflect a more general defect which results in decreased growth rate and heat stress response at lower temperatures than in the more virulent strains.

The regulation and a possible role of heat shock proteins has been also investigated at the gene level by Caruso et al., who cloned the hsp70 gene and studied its expression at different temperatures in different fungal isolates (18). This aspect will be discussed in the section on gene expression.

Relationship to Other Dimorphic Pathogens

The early biochemical events that occur during the mycelium to yeast phase transition in B. dermatitidis and P. brasiliensis are fundamentally similar to those reported for H. capsulatum (112). In both fungi, the triggering event in mycelium to yeast phase transition was a heat-related insult which led to various degrees of uncoupling of oxidative phosphorylation depending on the temperature of incubation. In both B. dermatitidis and P. brasiliensis, when mycelia were shifted to 37°C, respiration was coupled. In B. dermatitidis, respiration became completely uncoupled when the temperature used for transition was 43°C. In P. brasiliensis, a temperature of 41°C was sufficient to uncouple respiration during the phase transition. The temperature shift caused a decline in cellular ATP levels, respiration rates, and concentration of electron transport component (stage 1). The cells entered stage 2, during which respiration decreased or ceased (when temperature between 41 and 43°C were used). Finally, in stage 3, cells recovered and transformed to the yeast phases. The authors also showed that profound similarities exist in cell behavior during morphogenesis of these two fungi. In fact, in both B. dermatitidis and P. brasiliensis, in stage 2 under conditions in which respiration stopped, cysteine or other —SH-containing compounds activated the shunt respiratory pathways, which allows the utilization of mitochondrial substrates to provide energy to complete transition. At the same time, at least in B. dermatitidis, heat shock proteins are induced upon temperature shift (G. Shearer, G. Birge, G. S. Kobayashi, and G. Medoff. Annu. Meet. Am. Soc. Microbiol. 1985. F64, p. 375).

Relevance to Virulence

Because the temperature shift is so closely tied to virulence, genetic or physiological blocks to the transition take on special interest. To verify the hypothesis that the redox state of some critical —SH groups can control dimorphism, the effect of an —SH-blocking agent, p-chloromercuribenzenesulfonic acid (PCMS), on mycelium to yeast phase transition in H. capsulatum was studied (103). When mycelia were exposed to 100 μM PCMS for 24 h and washed free of the drug, they grew continuously at 25°C. These cells, though, when transferred to 37°C, never transformed to the yeast phase and eventually died after a few days. They also tested an —SH protector, dithiothreitol. In the presence of a 5 mM concentration of this drug, yeast transformation to mycelia did not occur (although when dithiothreitol was removed the transition occurred). It was argued that the effects of PCMS and dithiothreitol (which were not toxic to the cells) could be explained by postulating that some —SH groups most likely at the cell surface, were critical for determining the phase of H. capsulatum. In a more detailed analysis of the effect of PCMS, it was shown that PCMS-treated yeast cells could transform to mycelia at 25°C, but were unable to transform back to yeast cells at 37°C (113). The inability to transform to yeast cells at 37°C was permanent and irreversible despite multiple passages in media without PCMS.

So far, there has been no explanation for the specific function inhibited by PCMS, which prevents H. capsulatum mycelia from proceeding toward the phase transition. Blockage of cell differentiation by oxidation of —SH groups has also been demonstrated in other systems: in Friend erythroleukemia cells with PCMS (99) and during spore germination in Bacillus cereus (117). However, the effects on these systems were reversible when the —SH-blocking agent was removed from the culture. whereas the effects of PCMS on H. capsulatum were irreversible. It was suggested that PCMS might cure the cells from a plasmid or inhibit a chromosomal translocation (113) somewhat similar to that seen during yeast mating type (60) or in the variation of surface antigens in trypanosomes (62). The abortive mycelium to yeast transition of H. capsulatum is sufficient to provoke a protective immune response in animals and therefore is of potential therapeutic interest. This work posed a general question on the pathogenicity of dimorphic organisms in general: inhibition of functions sensitive to agents such as PCMS in other dimorphic pathogenic fungi or mutations in a gene involved in the transition, beyond the point of commitment, will cause the organism to die or block the differentiation process and serve as an efficient vaccinating inoculum. In fact, Medoff et al. (113) demonstrated that though there were no differences in the virulence of normal and PCMS-treated yeast cells in CD-1 mice. PCMS-treated mycelia did not convert to yeast cells in the same mice as shown by histopathological examination of tissues at necropsy. Increasing the concentration of PCMS-treated Downs mycelia could also increase significantly the survival of mice after infection with virulent yeast strains, and the increase in survival was proportional to the dose of the immunizing inoculum (Fig. 7).

In more recent work, Medoff et al. have shown that PCMS inhibits the mycelium to yeast phase transition of the virulent G184A and G222B strains (110). In this case, however, cultures were able to grow once transferred to 37°C, and only temperatures above 40°C prevented growth. Conversely, PCMS-treated Downs mycelia grew at 34°C. They concluded that, since mycelia can grow at high temperatures (37°C for pathogenic strains and only at 34°C for the less pathogenic Downs strain), the mycelium to yeast transition at high temperatures is not necessary for growth. This is an important notion since it implies that mycelium-specific genes can be expressed at any physiological temperature (compatible with cell growth) while at least some yeast-specific genes probably require high temperature (e.g., tem-
per temperature-dependent promoters) and a specific environment for the regulation of their expression.

This has indeed provided the first direct evidence that the mycelial phase of \textit{H. capsulatum} is nonpathogenic and that transition to the yeast phase is required for progressive infection.

**Other Factors Inducing Morphogenesis**

A number of compounds such as cyclic adenosine 3',5'- monophosphate (cAMP), or agents that interfere with intracellular levels of cAMP such as theophylline, and acetylsalicylic acid, prostaglandin \( E_1 \), or even a protein such as nerve growth factor have been described as agents that can induce yeast cells to transform to mycelia at \( 37°C \).

**cAMP and other substances.** In \textit{H. capsulatum}, the change from 25 to \( 37°C \) initiates a series of reactions leading to changes of the intracellular level of cAMP (103). The level of cAMP is about five times higher in mycelia than in the yeast phase. The change in the content of the cyclic nucleotide occurs during the yeast to mycelial transition at \( 25°C \) (114). Intracellular cAMP increased at the same rate in control cultures and yeast cells incubated at \( 25°C \) for the first 72 h. Subsequently, when yeast cells underwent elongation and branching, cAMP levels began to increase in transforming cells compared with that in controls. Changes in the level of cAMP were also detected extracellularly in the medium at 48 to 72 h after temperature shift. In the reverse transition from mycelium to yeast phase at \( 37°C \), changes in the intracellular level of cAMP were again observed; the cAMP concentration fell in transforming cells and in the growth medium. When buds began to emerge on hyphae, the cAMP content began to rise to levels characteristic of the yeast phase.

To determine whether changes that occur during normal temperature-dependent yeast to mycelium transition were manifestations of the phase change or simply a response to temperature shift, exogenous cAMP or inhibitors of cAMP phosphodiesterase were added to yeast cells at \( 37°C \) (103). Addition of 2 mM dibutyl cAMP (a more stable derivative of cAMP) or 5 mM theophylline to a yeast culture resulted in conversion to mycelia at \( 37°C \). Sacco et al. found similar results when acetylsalicylic acid, prostaglandin \( E_1 \), and nerve growth factor (the latter two hormones have also been related to cAMP action) were used (143). They found that the biochemical changes induced by these agents were the same as in the control, temperature-induced cells. In fact, the temporal order of changes was the same in both phase transitions: changes in respiration and in the activities of cystine reductase and cysteine oxidase occurred early, and the morphological changes and increase in cAMP occurred later.

Cyclic nucleotides have been found to play a key role in regulating cell differentiation in fungi and molds, but the mechanism of action is unknown in \textit{H. capsulatum}. It has been suggested that they may function by activating a cAMP-dependent protein kinase or a membrane receptor; in either case, cAMP would initiate in \textit{H. capsulatum} a series of events that result in phase transition. A possible mechanism by which cAMP may act through the polymerization of tubulin has been discussed (J. Medoff and G. Medoff, \textit{J. Cell Biol.} \textit{95}:44a, 1982). Another speculation links the regulatory changes of cAMP levels to other regulatory phenomena (103, 143). Since cAMP can induce phase transition without changing the temperature of incubation of the cells, other parameters such as the redox potential (regulated via cystine reductase) may act independently at the level of cAMP by regulating the activity of cAMP phosphodiesterase. The redox potential, governed by —SH groups, could then have a regulatory role in the level of cAMP, which in turn would...
control morphogenesis, arguing that a number of intralock-
regulatory circuits converge at cAMP.

Nucleic Acid Metabolism

RNA synthesis. Cheung et al. were the first to measure the
level of synthesis of total RNA in yeast and mycelial phases
and during morphogenesis in *H. capsulatum* (21). Total
RNA synthesized over an 8-h period was 3 to 12 times higher
in mycelia than in yeast cells grown in synthetic Salvin
medium (149), but the RNAs of both phases were indistin-
guishable (21, 48). Gross changes in RNA synthesis were
found during morphogenesis in *H. capsulatum*. In the first 24
to 40 h (stages 1 and 2) during the mycelium to yeast phase
transition, RNA and protein syntheses decreased to nonde-
tectable levels as respiration fell. It is reasonable to specu-
late that the fall in RNA and protein syntheses is a conse-
quense of the heat shock with the subsequent lack of available
energy in the cell (uncoupling of oxidative phosphory-
lation).

Very little is known about RNA metabolism in other
dimorphic pathogenic fungi. Ramirez-Martinez and Rod-
riguez did a preliminary descriptive study on RNA synthesis
and **mRNA** precursors during morphogenesis (133–135). Then
San-Blas and San-Blas, in a subsequent study, found a fall in uridine incorporation in the first 8 h of the yeast to
mycelium transition (151). This time corresponds to bud
formation during the early steps of morphogenesis to myce-
lium in this fungus. They also studied the effect of inhibitors
of RNA (50 μg of actinomycin D per ml) and protein syntheses
(0.5 μg of anisomycin per ml) and found that, while the late
stages of morphogenesis in either direction are affected,
yeast cells are able to transform, producing elongated cells
characteristic of the early stages of differentiation. They
believed that new RNA synthesis was required only later
during transformation, after 24 h, when synthesis started
again (152). Unless, possibly, preexisting mRNAs were
present and becoming available at the onset of the differen-
tiation process, it is difficult to interpret these experiments.

RNA polymerases. Profound changes in RNA synthesis
during morphogenesis in *H. capsulatum* may be the result of
modification or synthesis of a new set of RNA polymerases.
Boguslawski et al. and Kumar et al. characterized three
distinct activities in yeast cells and only one in mycelial cells
(8, 9, 11, 84), but Kumar et al. and McMllian et al. revealed
the expected three RNA polymerases in mycelia as well (83,
106, 107). The number of protein subunits in sodium dodecyl
sulfate gels, elution profiles in column chromatography,
inhibition by rifamycin derivatives (107) or RNA (106), and
salt and ion requirements (83) were similar to those for other
eucaryotic cells, but marked differences were found at the
level of α-amanitin sensitivity. In both phases of *H. capsul-
tatum* RNA polymerase II (polII) is most resistant and polIII
is most sensitive, whereas in other eucaryotes polII is most
sensitive and polI is most resistant (in *Saccharomyces cerevisiae*, polII is most resistant and polI is most sensi-
tive). Kumar et al. also found that rabbit amilasa generated
against polIII of the yeast phase detected major antigenic
differences between yeast and mycelial polIII, although the
other two polymerases showed similar or identical antigenic
determinants in the two phases (86). This was the first case
in which differences in specific properties of corresponding
RNA polymerases were noted in two stages of the same
organism. What these changes in RNA polymerases repre-
sents is unknown, but they may imply a primary role of the
RNA polymerases in effecting phase transition by control-
ing differential gene expression.

Similar but less extensive studies have been done with
RNA polymerases from *P. brasiliensis* from both yeast and
mycelial phases (151). In contrast to the extensive literature
on RNA polymerases, six activities in the yeast and three
activities in the mycelial phases have been described and
found to be temperature dependent. No other characteriza-
tion has been reported on the extra enzymatic activities.

**Ribonucleotide polymerase.** Boguslawski et al. have found
that the yeast, but not the mycelium, phase cells of *H.
capsulatum* contain a ribonucleotide-incorporating enzyme,
which was termed ribonucleotide polymerase (10, 13). This
DNA-independent enzyme incorporates any of the ribono-
cleotide triphosphates into short oligos (five to six residues
per oligo). This enzyme, which has also been purified and
found to have a molecular weight of 900,000, is similar to
other homologous proteins present in other organisms. Its
physiological role in the cellular metabolism of *H. capsula-
tatum* remains unknown.

DNA polymerase. Jaso-Friedmann and Boguslawski have
partially purified DNA polymerases from yeast and mycelial
phases of *H. capsulatum* (70). In each phase two distinct
classes of this enzyme have been identified with no signifi-
cant differences among pola and polβ from mycelia and
yeast cells. Similarly, pol β from both phases were essen-
tially identical. However, the two forms of the enzyme were
not equally sensitive to the oxidative effect of N-ethylmale-
imide: DNA pol II (with a molecular weight of 122,000) was
insensitive to the —SH group inhibitor, was unaffected by
0.2 M KCl, and had exonuclease activity; DNA polIII (with
a molecular weight of 196,000) was affected by these two
chemicals but had no nuclease activity. Measurements of
levels of DNA polymerase activity at various stages of cell
growth showed that polIII has greater fluctuation than polII.

Gene Expression

In recent years, a number of laboratories have focused on
elucidating the molecular mechanisms that regulate differen-
tial gene expression during phase transitions in pathogenic
fungi. Despite the discovery of the perfect state of *H.
capsulatum* (A. capsulatum), classical genetic studies are
technically very difficult to perform with this organism.

“Reverse genetics,” however, provides opportunities to
understand the mechanisms of spatial (septa formation,
elongation of mycelia, and regulation of cell division) and
temporal control of differentiative and phase-specific genes.

It is clear that phase transitions in dimorphic fungi (mycelium⇌yeast) involve alterations in the pattern of
gene expression, which leads to the expression of specific
regulatory genes. These genes, in turn, control changes in
many aspects of the metabolic processes of the cell and in
the morphology of the organisms. Recently, attempts have
been made to isolate members of both types of genes active
either in one phase only or early during a transition phase of
*H. capsulatum*. Central to analyzing the process of differen-
tiation in a dimorphic organism is understanding the process
by which the cell regulates differential gene activity. In
eucaryotes, at first instance, genes can be classified as
structural and regulatory. The former include those coding
for proteins and are the target of regulatory genes; the latter
include genes that can cause protein modification (glycosyl-
atation, phosphorylation, etc.) or determine the concentra-
tion of given proteins during the differentiation process. This
class of genes can exhibit both temporal and, in the case of
higher eucaryotes, spatial specificity. Recently, studies have been initiated in *H. capsulatum* to analyze both structural (e.g., tubulin and yeast-specific sequences) and potentially regulatory (heat shock genes) genes.

**Tubulin.** A number of substances have been shown to influence differentiation in *H. capsulatum*, regardless of the temperature of incubation. These compounds can, in fact, trigger morphogenesis, by passing the normal time course of gene expression which is induced, naturally, by temperature in the appropriate environment. cAMP is one of these compounds and, as in other fungi (*Mucor racemosus* [95] or *Candida albicans* [119]), when added to the culture medium, it induces transformation to the mycelial phase at 37°C (103, 114). cAMP may conceivably affect morphogenesis in *H. capsulatum* at the level of microtubule assembly (Medoff and Medoff, J. Cell Biol. **95**(4a), 1982). Indeed, 20 to 200 μM methyl benzimidazole 2-yl carbamate (an antimotic agent known to bind fungal tubulin and inhibit polymerization of tubulin monomers [25]) blocks, at 25°C, the yeast to mycelium transition. Of course, tubulin blockage is a fairly drastic treatment and could affect other biological processes; however, although the methyl benzimidazole 2-yl carbamate-treated cells do not transform, they continued to respire and grow at 25°C. These observations are consistent with the notion that microtubule organization plays a role in the differentiation process. Harris et al., with the aim to elucidate the role of microtubule assembly during morphogenesis in *H. capsulatum*, have cloned and characterized the genes coding for tubulin (G. S. Harris, E. J. Keath, and J. Medoff, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987; G. S. Harris, E. J. Keath, and J. Medoff, unpublished data; G. S. Harris, personal communication). These authors have reported the cloning of tubulin genes from a genomic library of *H. capsulatum* G217B. In contrast to other eucaryotes, in which several genes are present per haploid genome, *H. capsulatum* has only one single α-tubulin (TUB1) and one single β-tubulin (TUB2) gene. By analyzing the entire nucleotide TUB1 and TUB2 coding sequences, the authors provided evidence that these genes are interrupted by five and eight short introns, respectively, ranging from 42 to 123 nucleotides. Sequence analysis performed by these authors also showed the presence of conserved sequences with regions of the homologous genes of *Aspergillus nidulans* (104) and *N. crassa* (120).

Harris et al. have also studied the expression of TUB1 and TUB2 genes during morphogenesis (Harris et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1987; G. S. Harris, E. J. Keath, and J. Medoff, submitted for publication). By Northern (RNA) blot analysis, they have demonstrated that *H. capsulatum* G217B mycelia express three α-tubulin mRNAs 2,500, 2,200, and 2,000 nucleotides long, while the yeast phase cells lack the intermediate 2,200-nucleotide mRNA. Both α- and β-tubulin genes were shown to be constitutively expressed. These two genes produce, as shown by Western blot (immunoblot) analysis of mycelial and yeast proteins, one α- and one β-tubulin in the yeast phase of strains G217B and Downs, while in the mycelial phase two additional isoforms, αC- and βC-tubulin, were detected. Harris et al. have explained the presence of these extra forms of tubulins as due to posttranscriptional modification of the proteins, since in isoelectricfocusing these peptides are slightly more acidic than the "precursor" protein. They have argued that this modification might be due to phosphorylation or tyrosylation. Since these changes in tubulin mRNA expression correlate well with morphological changes, they believe that regulation of tubulin genes plays a definite role in the differentiation process of *H. capsulatum*. 

**yps-3, a yeast-phase-specific gene.** In an attempt to determine whether genes required for transition or for the maintenance of the yeast phase (which may be eventually be involved in virulence) exist, Keath et al. have identified several sequences specifically expressed in the yeast phase of *H. capsulatum* (74a, E. J. Keath, A. A. Painter, E. D. Spitzer, G. S. Kobayashi, and G. Medoff, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988 F34, p. 397). By using differential hybridization techniques, they isolated nine yeast-specific sequences from a G217B complementary DNA library. One of these genes, *yps*-3, was of nuclear origin. The clone contains a 1.85-kilobase (kb) fragment which hybridized to at least three polyadenylated yeast-specific transcripts. To determine whether *yps*-3 expression was under temperature control, Keath et al. monitored, by Northern blots, RNA synthesis in mycelia shifted from 25 to 37°C. The earliest time in which a transcript was detected was 2 to 24 h (1.05-kb mRNA), while the remaining two transcripts, 0.95 and 1.35 kb, were measurable at day 3 of morphogenesis. Therefore, it was concluded that, although the *yps*-3 is developmentally regulated, it is not under temperature or heat shock control.

An analogous study was carried out on the intermediate-virulent and thermotolerant G186B strain. In this case, only the 1.25-kb transcript was found after 24 h of phase transition and no 0.95- and 1.05-kb transcripts were ever detected. When the less virulent and temperature-sensitive Downs strain was analyzed, no hybridization was found when yeast cells and either 37 or 34°C induced mRNAs were probed with the *yps*-3 sequence. The lack of transcription was not due to the absence of the DNA sequence since the authors showed by Southern analysis that this sequence is indeed present in the Downs genome. This implied that the gene was not required for transition, since the Downs strain can transform to the yeast phase without expressing it, but the possibility that its product may be involved with pathogenicity cannot be ruled out.

Keath et al. (E. J. Keath, E. D. Spitzer, A. A. Painter, G. S. Kobayashi, and G. Medoff, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988 F33, p. 397) have also demonstrated that the 1.85 kb of DNA in *yps*-3 is *Histoplasma* specific. They have, in fact, screened several DNAs from pathogenic and nonpathogenic fungi with the *yps*-3 clone: such a sequence did not hybridize, under low-stringency conditions, to either an avirulent or a virulent strain of *B. dermatitidis*, to a clinical isolate of *C. albicans*, to *Saccharomyces cerevisiae*, or to two molds that morphologically resemble the mycelial phases of *H. capsulatum*, *Sepeedonum* and *Chrysosporum*. In contrast, it did hybridize to all *H. capsulatum* strains tested (Downs, G184B, G186B, G217B, and G222B) and to an isolate of *H. fajcinaminus*, an azoepitc variant of *Histoplasma* that produces diseases in horses and mules (155). This gene provides the first case of temperature-dependent mRNA induction for further molecular characterization, and it is a potentially powerful diagnostic tool for the identification and classification of *H. capsulatum* isolated from human and soil sources.

**Heat shock response.** The induction of the heat shock response has been postulated to play a role not only in thermoadaptation, but also in development (14, 96). "Developmental activation" is used to refer to all of those types of heat shock gene activation distinct from heat induction and that occur in a cell during development (6). Parasites, and dimorphic organisms in general, are particular cases of...
developmentally regulated heat shock protein expression: in fact, these organisms may exist in multiple hosts that have different body temperatures and exhibit distinct stages during morphogenesis. Temperature is a crucial factor that controls phase transition, but it is very difficult to distinguish the effect of temperature on morphogenesis from the induction of the heat shock response in these systems.

It seems most likely that the developmental control elements are located further upstream to the TATA box than the sites for heat shock control expression and that the regulatory proteins which confer developmental control are distinct from the heat shock transcription factor (6). Certainly, the increase in temperature (the “heat shock”) must be maintained constant here, contrary to the true heat shock response in other systems, in which temperature must be lowered to ensure survival. All of these characteristics, together with the consideration that phase transition leads to a pathogenic form of a parasite, make the study of the heat shock gene family, its regulation, and its biological significance for the organism important for the understanding of phase transition in pathogenic fungi. Heat shock genes are, however, among those which are induced very early during transition and may play a critical role in adaptation to the new environment for these organisms. At temperatures at which heat shock proteins are not induced (heat shock mRNAs are absent), the mycelium to yeast phase transition does not occur (Maresca, unpublished data).

hsp70. Caruso et al. have examined the transcription of the hsp70 gene in two strains of H. capsulatum (18). The gene for the 70-kilodalton heat shock protein was cloned from a cosmid genomic library of the Downs strain. A 3.3-kb PvuII fragment containing the H. capsulatum hsp70 gene was subcloned in pBR328 (pMB12) and was shown to hybridize with RNA of the expected size of 2.3 kb from heat-shocked cells, with only a low-level signal in unshocked cells. In each pathogenic strain, a single copy of the H. capsulatum hsp70 gene is seen, with the exception of the Downs strain which displayed an extra band (18). Also in common with many other systems, the H. capsulatum genome does not contain sequences related to hsp70 (hsc70, cognate genes) within ca. 70% nucleotide identity. Further investigation is needed to determine whether additional DNA polymorphism is present in other heat shock genes as well as in other regions of H. capsulatum strains isolated from patients and soil to see whether any correlation between degree of pathogenicity and specific DNA idiosyncrasies exists in the genome.

Caruso et al. have studied the optimum transcription of the hsp70 gene by the Northern blot technique (18). Two strains were analyzed: the less pathogenic and more temperature-sensitive Downs strain and the more virulent and temperature-resistant G222B strain. Maximal transcription of the hsp70 gene occurred at 34°C in Downs, while a temperature of 37°C was necessary to induce maximal transcription in G222B in the first 3 h after shift-up (Fig. 8). It is difficult to explain the difference in the regulation of expression of the hsp70 gene: it could be due, for example, to differences in cis or trans regulatory elements or to a more general temperature sensitivity of a specific strain. But these data showed mostly a correlation among temperature sensitivity, degree of virulence, and, in this case, amount of transcription of a specific gene. The authors have speculated that the enhanced temperature sensitivity of the Downs strain may be due to a low level of transcription of hsp70 genes as well as to other heat shock proteins (18). It is possible, in fact, that a major difference in the regulation of the activation of the heat shock response exists among strains with different virulence (low thermotolerance of less pathogenic strains), possibly due to a low level of induction of the “factor(s)” that regulates the transcription of heat shock genes.

A heat shock response also has been demonstrated in parasitic protozoa such as Trypanosoma brucei and Leishmania major, which shift from insect vector (22 to 28°C) and a homeothermic mammalian host (37°C). Van der Plouw et al. have proposed that heat shock genes may also be implicated in the differentiation of these vector-borne parasites (166).

The evidence suggests that heat shock proteins play an important role in diphasic organisms, in which the phase transition is an adaptive response to a higher temperature. It is not clear from the experiments thus far described whether the expression of this gene family is indeed part of the process of differentiation or is an epiphenomenon involved in adaptation to the new environmental temperature. Isolation of mutants impaired in the expression of members of this gene family or strains obtained by transformation with in vitro mutagen heat shock genes and reintroduced by gene conversion in the wild-type genome, as has been done in Saccharomyces cerevisiae (S. Lindquist, UCLA Symposium on Stress-Induced Proteins, 1988, Keystone, Colo.), may provide a convincing answer to the question of heat shock protein involvement in the transition.

hsp83. Another heat shock gene (hsp83) has recently been cloned and studied by Minchioti et al. (G. Minchioti, M. Sacco, and B. Maresca, Abstr. Annu. Meet. Soc. Ital. Microbiol. Gen Biot. Microbicite 1988, p. 85). The coding sequence of H. capsulatum hsp83 closely resembles that of the Saccharomyces cerevisiae sequence (>70% homology in protein sequence). As shown for Drosophila sp., the maturation of the hsp83 gene is under temperature control. At “extreme” temperature (37°C for the Downs strain and 41°C for G217B), the mRNA is not properly spliced, but splicing activity could be maintained if heat shock protein synthesis was induced at an intermediate temperature before the shift to extreme temperature.

THE CELL WALL AND MEMBRANE

Cell Wall

Fungi in general have an outer rigid layer of polysaccharides that covers the fungal plasmalemma. It is the specific composition of the cell wall which determines the shape of the fungus: this is clearly seen when protoplasts are obtained from either mycelia or yeast-shaped cells, since upon enzymatic digestion of the outer cell wall a spherical protoplast is invariably obtained. Therefore, some fundamental characteristics of fungal physiology, such as apical growth, cell form, morphogenesis, and host-fungus interactions, have been attributed to specific determinants present in the cell wall composition (161).

The lack of peptidoglycan, glycerol, or ribitol teichoic acids is a fundamental difference in the composition of bacterial and fungal cell walls which is responsible for the resistance of fungi to antibiotics such as penicillin that interfere with bacterial cell wall synthesis. On the contrary, fungal cell walls contain α- and β-glucan and peptidomannans. Further, the integrity of the fungal cell wall is secured by chitin fibrils, which represent an important constituent during budding formation. Each fungus has its own specific cell wall components of particular macromolecular composition. An extensive literature exists on cell wall composition, antigenic properties, fungal growth, etc., of different
dimorphic fungi; refer to references 2, 3, 54, 126, and 139 for details. Here we discuss only those that may be important in relationship to phase determination.

Kanetsuna et al. have shown that, during phase transition from yeast cells to mycelia, the synthesis of α-glucans decreases rapidly (74). They assumed that this activity is temperature dependent, having a lower activity at 20°C than at 37°C. Further, they found fivefold more protein disulfide reductase activity during the mycelium to yeast phase transition and greater β-glucanase activity in mycelia. Such disulfide reductase activity was similar to that found by Nickerson and Falcone for Candida sp. (118). According to Kanetsuna et al., the morphological changes that occur during morphogenesis are due to specific cell wall components (74). This hypothesis is based on previous findings by Bartnicki-Garcia and Lippman, who found regional differences in the synthesis of the cell wall in Mucor rouxii (2, 3). According to their model, a simultaneous and equal synthesis occurs over the entire cell wall, producing a spherical yeast cell, while a specific synthesis in an apical region yields a cylindrical shape typical of mycelial morphology. Bartnicki-Garcia and Lippman had speculated that induction of β-glucanase activity (alone or in combination with disulfide reductase) causes a decrease in cell wall rigidity of a yeast cell, thus producing the elongation or budding (2, 3). At low room temperature, the production of β-glucan would be
favored, thus favoring mycelial growth, while a shift to 37°C, which favors α-glucan synthesis and low β-glucan synthetic activity, would produce yeast cells.

Contrary to what was reported by Kanetsuna et al. (74), other authors have shown that the synthesis of α-(1,3)-glucan is not "responsible" for yeast morphology (152). These authors have proposed a new model for dimorphism in P. brasiliensis based on the assumption that a spherical yeast form is produced by the simultaneous synthesis of the entire cell wall and that mycelia are produced when apical synthesis occurs. In addition, considering that during the early period of yeast to mycelium transition RNA and protein synthesis stop, they have postulated that the enzyme responsible for the synthesis of β-(1,3)-glucan changes its quaternary structure as a response to the change of temperature of incubation. In other words, they postulated the existence of interchangeable active "infective" forms of the enzyme, due to temperature activation or repression (58) or to the presence of an activator or a repressor molecule(s). In such a way they could explain the new protein activity formed in the absence of RNA or protein synthesis. Similarly, in the opposite transition, from mycelium to yeast phase. San-Blas and San-Blas (152) believe, as proposed by Kanetsuna (73), that the concomitant action of β-glucanase and protein disulfide reductase activities causes loss of cell wall rigidity and change in the shape of the organism. The cytoplasmic pressure on the softened cell wall would then be the "internal driving force" to initiate budding.

A number of investigations are reported in the literature on cell wall composition of both mycelial and yeast phases of H. capsulatum (26, 28, 79). These studies have reported differences in the composition of the cell wall and have divided different strains into chemotypes I [which has a large amount of glucan which is β-(1,3) linked and a small amount which is β-(1,4) linked] and II [characterized primarily by a α-(1,3) linked glucan with some β-(1,4)-linked glucan present]. Both H. capsulatum and B. dermatitidis produce glucanase and glycosidase in either phase. For cell growth, budding, and during morphogenesis, there should be a balance of enzymes responsible for the synthesis and lysis of the cell wall. Such enzymes are thought to be produced within specialized vesicles of the endoplasmic reticulum (139). From a quantitative point of view, there are discrepancies between isolates as determined by different laboratories. However, it has been shown for P. brasiliensis (150) that gross differences are present in the cell wall composition among strains with different levels of pathogenicity (127). Such differences are claimed to be important in determining antigenicity and virulence. Recently, Klimpel and Goldman have characterized cell wall constituents of an avirulent variant of H. capsulatum lacking α-(1,3)-glucan (77). They had initially isolated a spontaneous avirulent variant (G186AS) from a virulent strain of H. capsulatum (76). Two clones (G186AS and G186AR) were derived from strain G186A and shown to have comparable growth rates and total protein profiles on sodium dodecyl sulfate gel. The authors characterized cell wall components and found that the avirulent G186AS strain contains about 1,000-fold less α-(1,3)-glucan than its virulent parents. They have no explanation for the relationship between the difference in glucan content and the level of virulence, but suggested that this polysaccharide may have a structural function, providing a proper substrate for proteins involved in virulence.

**Membranes**

Virulence of fungi has been associated with ability to transform to a pathogenic yeast morphology. To understand host responses against fungal infections, studies dealing with the characterization of antigens involved in the immune reactions have been done, since most immunogenic antigens are invariably present in the cell membranes (85). Furthermore, it has been shown that fungal membranes carry complement-binding proteins (30; B. V. Kumar, personal communication), and the possible preparation of antifungal vaccines by using specific membrane proteins makes it important to obtain pure membranes to characterize membrane antigens (113). The results in H. capsulatum are in agreement with other studies that indicate a role for plasma membrane in biological processes in lower as well as higher eucaryotes, such as in development, tissue organization, immune response, and recognition of external messages (132).

A method of purification of plasma membranes uses a discontinuous sucrose density gradient, which was employed for both mycelial and yeast phase membranes (81). A comparison of two fractions revealed quantitative and qualitative differences in the expression of several membrane-specific enzymatic activities as well as physical differences upon observation by electron microscopy. The difference in the appearance of the membrane vesicles in two distinct density bands (in yeast cells, 72% of radioactive 32P was associated with the 0 to 30% sucrose band, while in mycelia, >98% was associated with 40 to 50% sucrose) may result from different lipid or carbohydrate composition of the two membrane preparations. This would then be consistent with earlier observations of marked differences in sensitivity to amphotericin B, which depends on the sterol content of the cellular membranes (20, 22, 109).

**PAST AND FUTURE PERSPECTIVES**

Many dimorphic pathogenic fungi have been used as model systems to study development and differentiation in lower eucaryotes and to investigate host-parasite interaction. Despite the fact that the differentiation process can be easily induced under laboratory conditions and can, in theory, be analyzed, dimorphic pathogenic fungi have presented a number of problems: slow growth rate, difficulties in growing clones from single cells, and the induction of synchronous growing cultures have added technical problems which have not been solved completely. In addition, thinking of reversible shifts from mycelia to yeast cells as a simple change from a particular cell wall composition to another (e.g., α-(1,3)-glucan ⇔ β-(1,3)-glucan) may be too simplistic. These models do not explain the number of complex changes that occur during the transitions. Perhaps the processes may be more analogous to the differentiative system seen in Bacillus subtilis sporulation. The bacterium Bacillus subtilis undergoes differentiation from a vegetative to a sporulation cycle: during this process, a prespore cell develops, via a series of intermediate morphological steps, into a dormant highly resistant spore. The nature of the mechanisms that determine cell differentiation in Bacillus subtilis has been largely proven now to result from the specific induction of spatially and temporally active control genes. Distinct pathways leading to gene activation or repression have been established in each cell stage, and, further, most of the genes controlling development have now been cloned (34, 35). Regulatory genes such as the nine...
that eventually lead to the new phase of the organism. On the contrary, a large number of genes activated during phase transition may code for important functions unrelated to the developmental process. One may envisage “groups” of genes present in the genome of these dimorphic fungi which are coordinately activated for adaptation to the new conditions and specific sets of regulatory and phase transition genes.

Available data also suggest that a specific “checkpoint(s)” exists in *H. capsulatum* during the transition to yeast transition which allows the organism to determine whether or not it is really in the new environment: for example, a rise in temperature should not simply be due to circadian fluctuations in the external environment (night or day, exposure to shade or sun) but determined by host invasion. Therefore, a change in the temperature should not be “read” by the fungus as the necessary and sufficient condition to start the differentiation process. Temperature would initiate a series of reactions that permit the organism to proceed towards a new cell stage only when other conditions, such as the presence or absence of growth factors, redox potential, etc., are satisfied.

A comprehensive view of the key interactive gene groups in the differentiation process in *H. capsulatum* is still lacking, but new information is forthcoming. As described above, at least two complex sets of modifications occur during the shift from mycelia to yeast cells: profound changes in the mitochondrial activities and the induction of the heat shock phenomenon. It appears that these molecular changes represent “unit circuits,” each one dealing primarily with a “single” phenomenon: the effect of temperature on mitochondrial electron transport efficiency and energy synthesis or the induction of heat shock genes. These physiological genetic compartments may not themselves be instructive in the regulation of specific patterns of gene expression, but they may be critical for the adaptation of the organism to the new environmental temperatures at which patterns of gene expression take hold. Progress toward the late stages of morphogenesis would be contingent on the circuits, but not result from them. Thus, it is not surprising that many circuits may be activated in both mycelia and yeast cells and not necessarily only during morphogenesis. For example, depending on the exact temperature used to induce mycelium to yeast phase transition in *H. capsulatum* (as well as in *B. dermatitidis* and *P. brasiliensis*), mitochondrial activity varies enormously, from a slight decrease in metabolic activity to a complete block in the electron transport. Similarly, the level of expression of heat shock genes varies according to the temperature. In these cases, either a mild or an extreme temperature insult permits the completion of the phase transition.

In other words, a temporal superimposition of functions necessary for survival to the new life conditions occurs concomitantly with the pattern of gene expression directly involved with morphogenesis. Another example of this phenomenon is given by changes in the metabolism of cysteine utilization during phase transition. —SH compounds appear to have at least two functions. In their presence, and in a condition of extreme heat shock, shunt pathways are activated and bypass the block in the cytochromes that occurs in stage 1 of the conversion of mycelia to yeast phase. The discovery that virulent, more temperature-resistant strains induce the shunt only at higher temperature (39 to 43°C) would exclude this phenomenon as a key step towards phase transition. Rather, the induction of the shunt pathway may represent an alarm signal triggered at high temperature or
stress. Therefore, the function of cysteine in the protection of mitochondrial respiration is apparently critical for survival at physiological temperature only in those temperature-sensitive strains such as Downs, and it is not indispensable for the phase transition itself. Such a view may rationalize some of the discrepancies in the cysteine requirement described by several authors, for it is likely that strains with different levels of thermotolerance (and virulence) may have been used in their studies. That the shunt pathway can be induced in both mycelial and yeast cells supports the hypothesis that this function has a role during the normal cell growth if 'stressful' conditions similar to a severe heat shock are encountered by the cells.

Assuming that mycelial morphological characteristics are advantageous for saprobic growth, and the yeast morphological characteristics are advantageous for parasitic growth, we can speculate that the requirement for cysteine to complete the transition from mycelium to yeast phase in temperature-sensitive strains is an important biological control mechanism for *H. capsulatum*. The requirement of cysteine in stage 3 can be explained by the fact the physiological habitat in which the yeast cells must survive is a very specialized environment inside macrophages with high reducing capacity. Therefore, the shift in temperature from 25 to 37°C converts the organism from a prototroph to a cysteine auxotroph. Teleologically, the requirement for cysteine late during the transition and for the maintenance of the yeast phase could assure that random temperature increases will not result in morphological conversion and that yeast phase growth will occur only in an appropriate host environment in which exogenous cysteine is available. An interesting observation consistent with this notion is that binding of PCMS, presumably to the cell membrane, irreversibly blocks the mycelium to yeast transition at some point between stages 2 and 3 (113).

Morphogenesis also involves profound modifications in the expression of specific genes. How temperature intervenes in gene expression is not yet clear. Somehow a signal must be transduced from the environment to the nucleus. This "signal" may be converted into a chemical message at the membrane level. Several laboratories have described early biochemical events that occur after the shift-up in temperature. These include changes in cAMP levels and the activation of the heat shock response. Both events have been suggested to play a role in cellular differentiation: in *H. capsulatum* it has been postulated that changes in temperature regulate the redox state of the cell and, in turn, the cAMP level via the adenyl cyclase-cAMP phosphodiesterase activities. cAMP would then be, among other regulatory functions, responsible for the polymerization of tubulin, which has been described as one of the important steps during morphogenesis in this fungus. Furthermore, heat shock proteins may play a role in cell differentiation. Present models postulate that the transducing signal(s) from temperature variation down to the level of DNA occurs through the activation of a heat shock transcription factor which, in the case of *Drosophila* and *Saccharomyces* spp., has been shown to be modified by phosphorylation. It has not been demonstrated how a specific phosphorylase kinase activity is regulated by a temperature increase, but it has been shown that a decrease in the level of a CAMP-dependent phosphor ylase in response to the synthesis of heat shock proteins and acquisition of thermotolerance in *Saccharomyces cerevisiae* (157). It is tempting to speculate that in *H. capsulatum* the modification in cellular metabolism after temperature shift causes changes in the level of cAMP which may (in addition to polymerization of tubulin and regulation of other activities) be linked to the regulation of heat shock gene activation.

In addition, it has been suggested that heat shock genes may be activated by a "master regulator," an inducible factor which permits the subsequent activation of heat shock transcription factor (96): the activity of the master regulator is presumably specifically modulated in the strains with different thermotolerance. The different level of active heat shock transcription factor capable of activating the heat shock response would be responsible for the capacity of a strain both to survive at a given temperature and to proceed towards phase transition.

These considerations reflect the view that dimorphism in fungi is a complex mechanism that includes, in addition to changes in the regulation of genes directly involved in morphogenesis, modifications in the regulation of genes coding for functions necessary for the adaptation to the new living conditions and for the induction of pathogenicity.

An important consideration from an evolutionary point of view is that, in nature, only a small number of mycelia or microconidia that infect humans will go through the transition that produces the yeast phase. This characteristic poses major questions. How can developmentally regulated and phase-specific genes (including those coding for virulence) be maintained in a fungal population without an external selective pressure? Do these genes have different functions in the mycelial phase? Can these genes code, in the mycelial phase, for functions necessary for survival and therefore be uniformly maintained in the genome of these fungi? Study of the role of the heat shock response in dimorphic fungi may suggest that genes coding for functions critical for survival in one cell phase may be used during the differentiation process as well and could explain how transitional specific genes are maintained in organisms with a facultative growth phase. Further unresolved questions include the following. What determines the nature of the mechanism of gene expression in dimorphic fungi? How can changes in the temperature of incubation (or in the redox potential) activate or repress specific genes? Is there a specific sequence of events under the control of a small number of genes that determine virulence in fungi? What are the fungal and cellular determinants of infection? What genetic mechanisms generate different levels of pathogenicity in various isolates? What determines the change from infection to disease?

We cannot overlook issues which may be secondary in mechanistic studies but are of potential practical importance. Study of the regulation of the heat shock genes is therefore also interesting from a medical point of view, and stress proteins should be considered among candidates for specific vaccines (as shown by the study that led to the isolation of transitional avirulent PCMS strains which maintain the ability to serve as immunogens in mice) (113).

The phenomena that occur after a shift in temperature tie in interlocking chains of steps, and in some measure it is possible to interfere with pharmacological, biochemical, or physical agents, with the course of differentiation bypassing steps. It is possible that the very early steps may follow a linear sequence of events (as proven by the addition of aCAMP, which forges yeast cells to mycelia even at 37°C), after which the pattern of gene expression may follow multiple paths. Thereafter, a single chemical compound could not mimic the natural progressive pattern of gene expression. This implies that it is possible to induce differentiation with a single compound only at the very early...
FIG. 9. Sequences of events associated with mycelium to yeast transition in the Downs strain of *H. capsulatum*. Events that are boxed in are related. Respiratory activity is indicated by the bold line. These events also can be activated in either mycelia or yeast cells without inducing phase transition.

moments of the process, and not later, and that a single compound may have complex but definable sequela.

Reverse genetics is now primed to analyze key steps of the differentation process. Changes in the regulation of gene expressions (heat shock genes, *yps-3*, and tubulin), in enzymatic activities (enzymes involved in cysteine metabolism, mitochondrial functions, etc.), and in RNA and protein synthases have all been suggestive thus far. The models of differentation of dimorphic fungi are vague (Fig. 9), and much more work has to be done. Cloning and characterization of specific genes activated during phase transition and the elucidation of the function of critical gene products must be performed. Specifically, an analysis of early genes coding for regulatory functions, as well as studies of mutants and transfection analysis of in vitro generated mutations of putative regulatory genes, seems the best route now toward understanding the regulatory induction of genetic pathways that lead to phase transition and possibly the mechanisms that induce virulence in dimorphic pathogenic fungi.

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LITERATURE CITED


DIMORPHISM IN H. CAPSULATUM


Voi-Salvin, 147.