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# Isolation and Characterization of *Candida albicans* Morphological Mutants Derepressed for the Formation of Filamentous Hypha-Type Structures

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**Several *Candida albicans* morphological mutants were obtained by a procedure based on a combined treatment with nitrous acid plus UV irradiation and a double-enrichment step to increase the proportion of mutants growing as long filamentous structures. Altered cell morphogenesis in these mutants correlated with an altered colonial phenotype. Two of these mutants, *C. albicans* NEL102 and NEL103, were selected and characterized. Mutant blastoconidia initiated budding but eventually gave rise to filamentous hypha-type formations. These filaments were long and septate, and they branched very regularly at positions near septa. Calcofluor white (which is known to bind chitin-rich areas) stained septa, branching zones, and filament tips very intensely, as observed under the fluorescence microscope. Wild-type hybrids were obtained by fusing protoplasts of strain NEL102 with B14, another morphological mutant previously described as being permanently pseudomycelial, indicating that genetic determinants responsible for the two altered phenotypes are different. The mutants characterized in this work seemed to sequentially express the morphogenic characteristics of *C. albicans*, from blastoconidia to hyphae, in the absence of any inducer. Further characterization of these strains could be relevant to gain understanding of the genetic control of dimorphism in this species.**

It is widely admitted that the pathogenicity of *Candida albicans* must be at least partially based on the capability of blastoconidia (oval yeast cells) to form mycelial filamentous structures that the host phagocytic system cannot cope with (reviewed in reference 14). This capability is well expressed in vivo as the organism is normally observed in invaded tissues in its hyphal form (18). It is also readily manifested in vitro through the action of a complex set of chemical inducers and environmental factors that provide the most essential characteristic for the differentiation of *C. albicans* from other *Candida* species (8). The cytological (4, 21, 22) and biochemical (19) details of the process of formation of filamentous structures (mycelium, pseudomycelium) have been analyzed. Electrophoretic patterns (23), enzyme activities (12), or immunosera (either polyclonal or monoclonal) (2, 16, 24) were used to identify specific gene products of either blastoconidia or hyphae. However, contrary to the initial expectations, the drastic changes in morphology are not correlated by dramatic changes in the proteins expressed (11).

The cellular controls that operate to regulate these changes, i.e., the role of differential gene expression and the possibility of dimorphism-specific genes, are very far from being elucidated (20). We believe that genetic strategies to analyze dimorphism of *C. albicans* should eventually lead to definitive answers and enable us to develop a model that could account for all known facts regarding the morphological transition. The rational use of genetic strategies in *C. albicans* research was prompted by the demonstration of diploidy (26) and the introduction of methods to induce and characterize mutants in this species (6, 7, 25). The application of molecular genetic tools to this system, including the isolation of specific mutants by gene disruption, has also been made available (10). Therefore, the question of genetic control of dimorphism in *C. albicans* can be addressed.

Several types of dimorphism-altered mutants have been isolated and described (1, 3, 5, 13, 15). In this communication, we describe what seems to be a new type of morphological mutant; blastoconidia of these new mutant strains produced long filamentous structures with a regular branching pattern in the absence of any specific inducer of germ-tube formation.

## MATERIALS AND METHODS

**Organisms and culture conditions.** *C. albicans* 1001 (also ATCC 64385) was obtained from the Spanish Type Culture Collection (Department of Microbiology, University of Valencia, Valencia, Spain). *C. albicans* B14 ($Y^-$ $His^-$) was described previously (3) as a permanent filamentous mutant strain, mostly of pseudomycelial appearance ($Y^-$ phenotype). *C. albicans* EL2, NEL102, and NEL103 were isolated as part of this work as described below. YED medium and YED agar, which were used as complete media, minimal medium (MM agar), supplemented when appropriate with various amino acids or other growth factors, and maintenance of stock cultures were as previously reported (3). Germ-tube formation in the wild-type strain was induced by incubating yeasts in a medium (YE-Pro) containing 0.1% yeast extract and 0.01% proline.

**Mutagenesis and isolation of mutants.** Given the diploidy of *C. albicans*, the basic mutagenic process consisted of treatment with nitrous acid to induce a high number of mutations, followed by gentle UV irradiation to unmask some of the recessive traits by mitotic recombination (6). A total of $10^9$ exponentially growing cells were collected and washed twice with 5 ml of 0.1 M sodium acetate buffer (pH 4.5). Cells were pelleted, suspended in 2 ml of 0.06 M sodium nitrite in the same buffer, and incubated at 28°C for 25 min in a water bath with gentle shaking. Mutagenesis was stopped by adding 33

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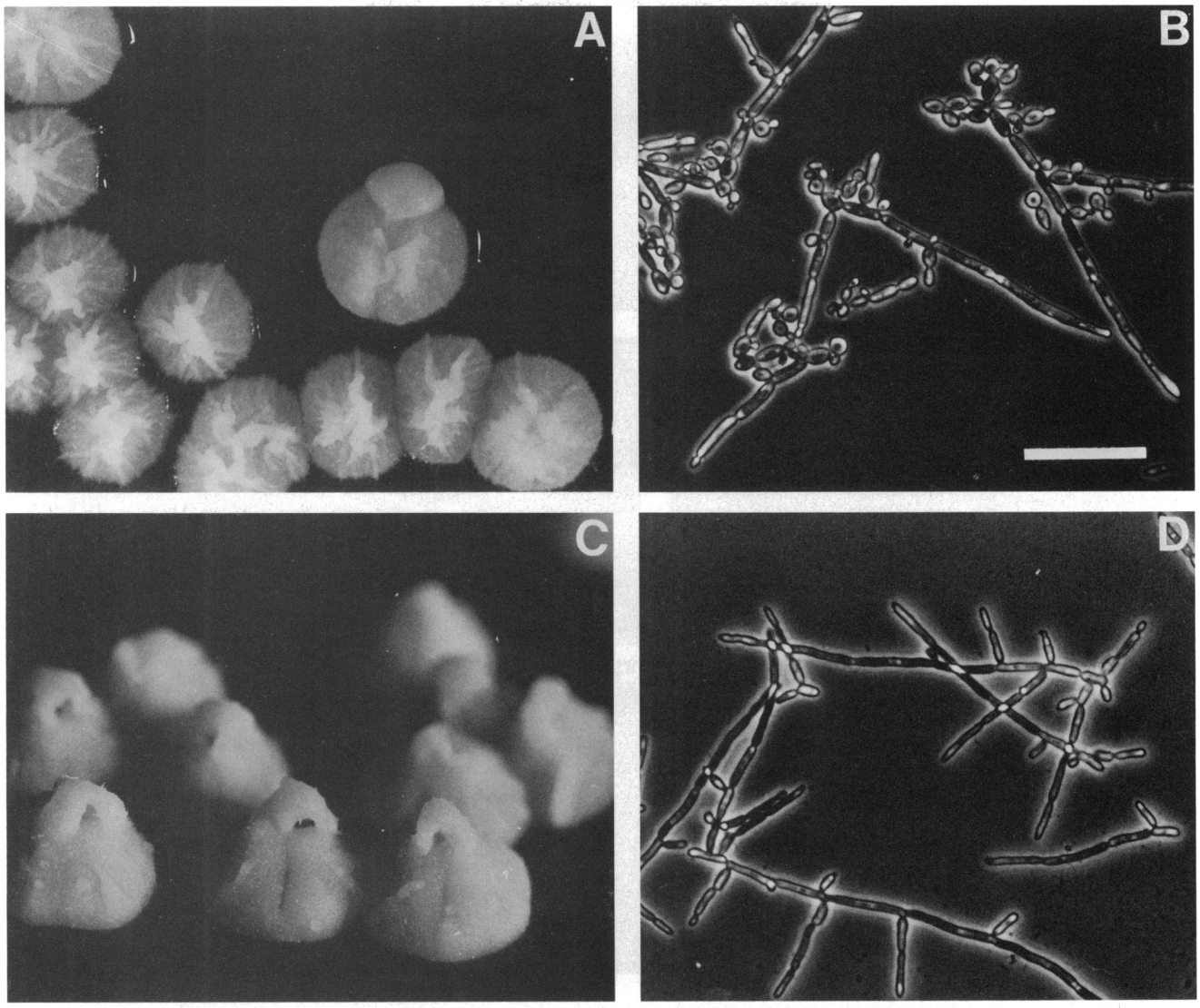


FIG. 1. Colonial and cell morphologies of *C. albicans* NEL102 (A and B) and NEL103 (C and D). Bar, 40 μm.

ml of 0.1 M sodium phosphate buffer (pH 7.5), and appropriate dilutions were plated in YED agar. The plates were irradiated for 40 s with a UV lamp at a dose of 16 erg/mm$^2$ per s as measured with a dosimeter (Ultraviolet Products Inc.), incubated at 28°C, and processed after 5 days. Viability after the nitrous acid treatment was approximately 0.3%, and it was reduced to 0.01% by the additional UV irradiation. In some cases, UV treatment was applied to cell suspensions instead of the plated cells. Mutants were isolated by treating the wild-type strain 1001 and obtaining several auxotrophs, among them strain N10 (Ade$^-$). A new round of nitrous acid and UV irradiation applied to N10 enabled us to obtain *C. albicans* EL2 (Ade$^-$ Arg$^-$). The double auxotroph was the starting point for mutagenesis and isolation of morphological mutants.

**Complementation analysis by protoplast fusion.** Strains B14 and NEL102 were hybridized by fusing protoplasts (6) obtained from exponentially growing cells by the procedure previously described (3). Fusion products were selected by regeneration in the absence of growth factors, and the hybrids were obtained by transferring these products to MM agar.

**Phase-contrast and fluorescence microscopy.** Cells were observed and photographed with a Nikon phase-contrast microscope equipped with an Optiphot system. For calcofluor fluorescence staining, cell suspensions in distilled water were mixed with an equal volume of a solution containing 25 mg of calcofluor white (American Cyanamid Co., Bound Brook, N.J.) per ml.

## RESULTS

**Isolation of morphological mutants.** To isolate new types of morphological mutants, we used a double-enrichment procedure based on the filtration of suspensions of mutagenized cells that had been allowed to express any phenotypic alteration in morphogenesis. The process began with the double-auxotrophic mutant strain *C. albicans* EL2 (Ade$^-$ Arg$^-$), which did not show any alteration in morphogenesis but grew as blastoconidia and gave rise to smooth colonies in

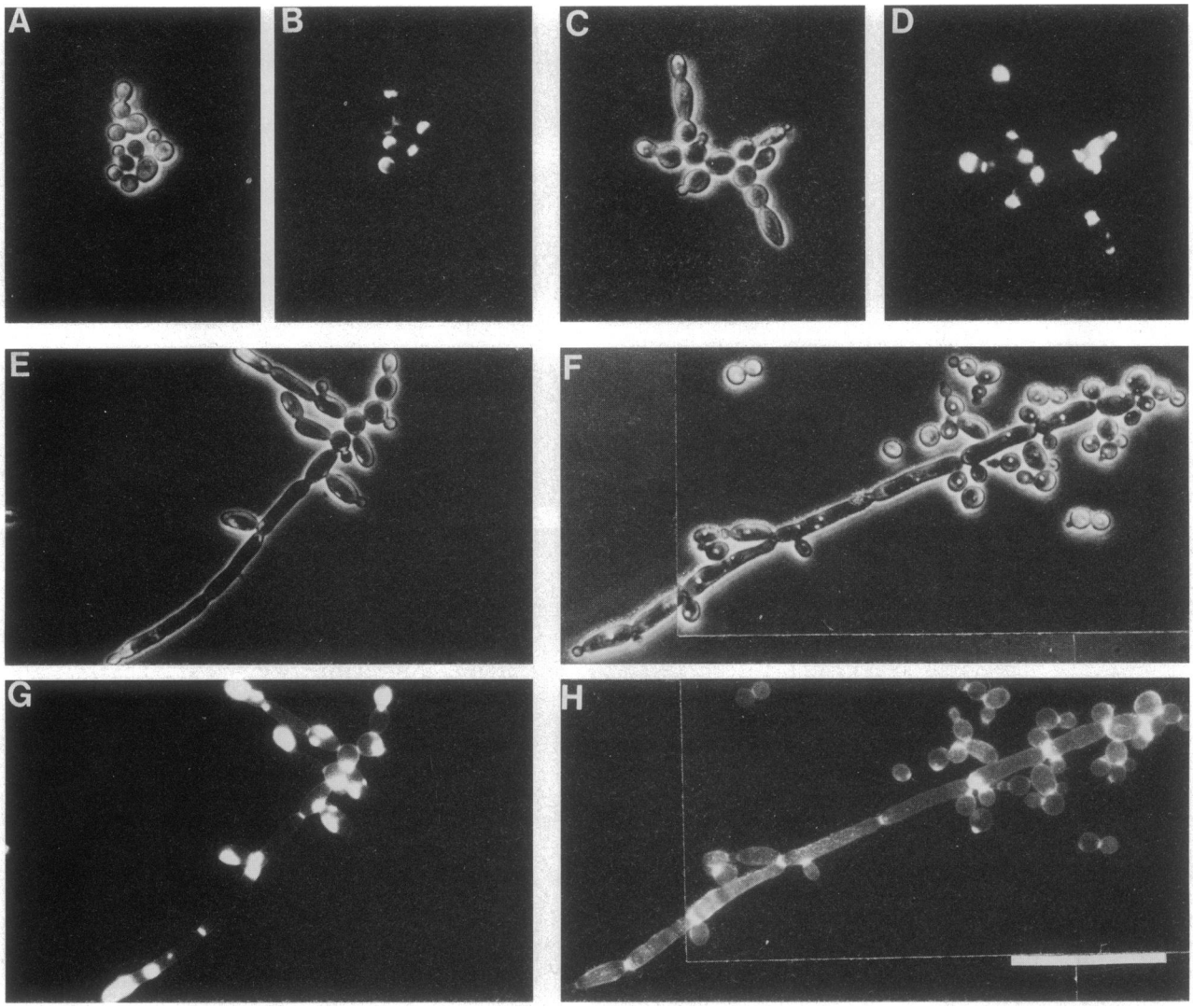


FIG. 2. Phase-contrast and calcofluor fluorescence-stained micrographs of *C. albicans* NEL102 cells grown for different times in YED liquid medium. (A and B) 0 h; (C and D) 3 h; (E and G) 6 h; (F and H) 26 h. Bar, 40 μm. (To show the long filamentous structures, micrographs F and H are composites of two microscopic fields.)

YED agar. It was convenient to start with a strain carrying two genetic markers for an unequivocal differentiation of any mutant from potential contaminants appearing during the manipulations and to have some genetic traits that could be useful for further characterization of the new mutants by protoplast fusion. Cell suspensions of strain EL2 were treated with nitrous acid and UV (as described in Materials and Methods) and grown for 3 h in YED medium to allow for the expression of any morphological phenotype. A first enrichment was done by sedimenting the cells, suspending them, and filtering them through filter paper. Most of the oval cells were filtered, whereas filamentous structures were retained. The filter with the retained cells was placed in a flask containing YED medium for a new incubation of 4 h at 28°C. The suspension was then filtered, and the retained cells were washed out of the filter and placed on YED agar. The plates were incubated for 5 days and screened for colonies with characteristic morphologies, such as a rough or hairy aspect, which is typical of mutants growing mostly as filamentous structures. A total of 5,800 colonies were screened, and 18 of them were selected as potential candidates by their rough colonial appearance, clearly distinguishable from the smooth wild type. Microscopic examination of the cells of those colonies revealed that cells from 9 of the 18 clones had a clear filamentous structure. We finally selected strains NEL102 and NEL103, the mutant strains that exhibited a more homogeneous morphology with regard to the filamentous forms observed in cultures, for further characterization.

**Characterization of two morphological mutants.** Colonies of *C. albicans* NEL102 displayed a characteristic irregular wrinkled hairy aspect (Fig. 1A) and consisted mainly of long filamentous structures with occasional oval forms that seemed to emerge from them (Fig. 1B). On the other hand, strain NEL103 gave rise to colonies with a very characteristic top and an apparently granular surface; again, almost only filamentous forms were observed in the corresponding microscopic preparations (Fig. 1C and D). These morphologies were consistently maintained through repeated subcultures in complete and MM medium supplemented with

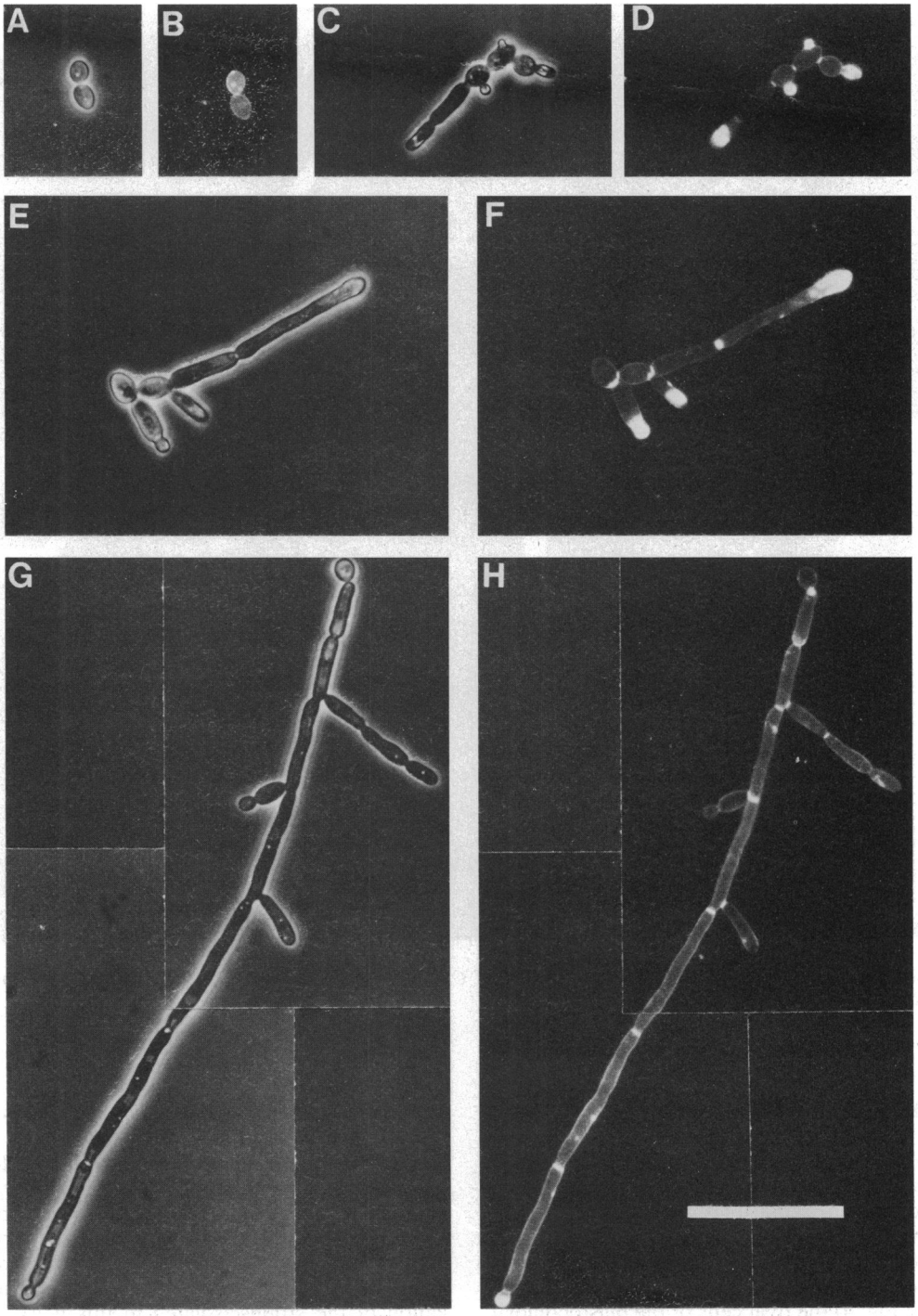


FIG. 3. Phase-contrast and calcofluor fluorescence-stained micrographs of *C. albicans* NEL103 cells grown for different times in YED liquid medium. (A and B) 0 h; (C and D) 3 h; (E and F) 7 h; (G and H) 24 h. Bar, 40 μm. (To show the long filamentous structures, micrographs G and H are composites of two microscopic fields.)

adenine and arginine, the two growth factors for which the strains were deficient.

Oval yeast cells were obtained by filtering 24-h-old liquid cultures of both mutants and incubated in YED medium at 28°C with shaking for a detailed examination of their patterns of growth in liquid cultures. Samples of these cultures were obtained at different times, stained with calcofluor white, and observed and photographed under the microscope, both with the phase-contrast system and with the fluorescence device. Both strains NEL102 and NEL103 behaved similarly under these conditions (Fig. 2 and 3). Growth of blastoconidia seemed to be initiated by budding, but the corresponding buds did not give rise to independent oval cells but remained as chains of unseparated cells with very clear constrictions, showing the appearance of pseudomycelial structures. Cultures of 6 to 7 h (Fig. 2E and G and 3E and F)

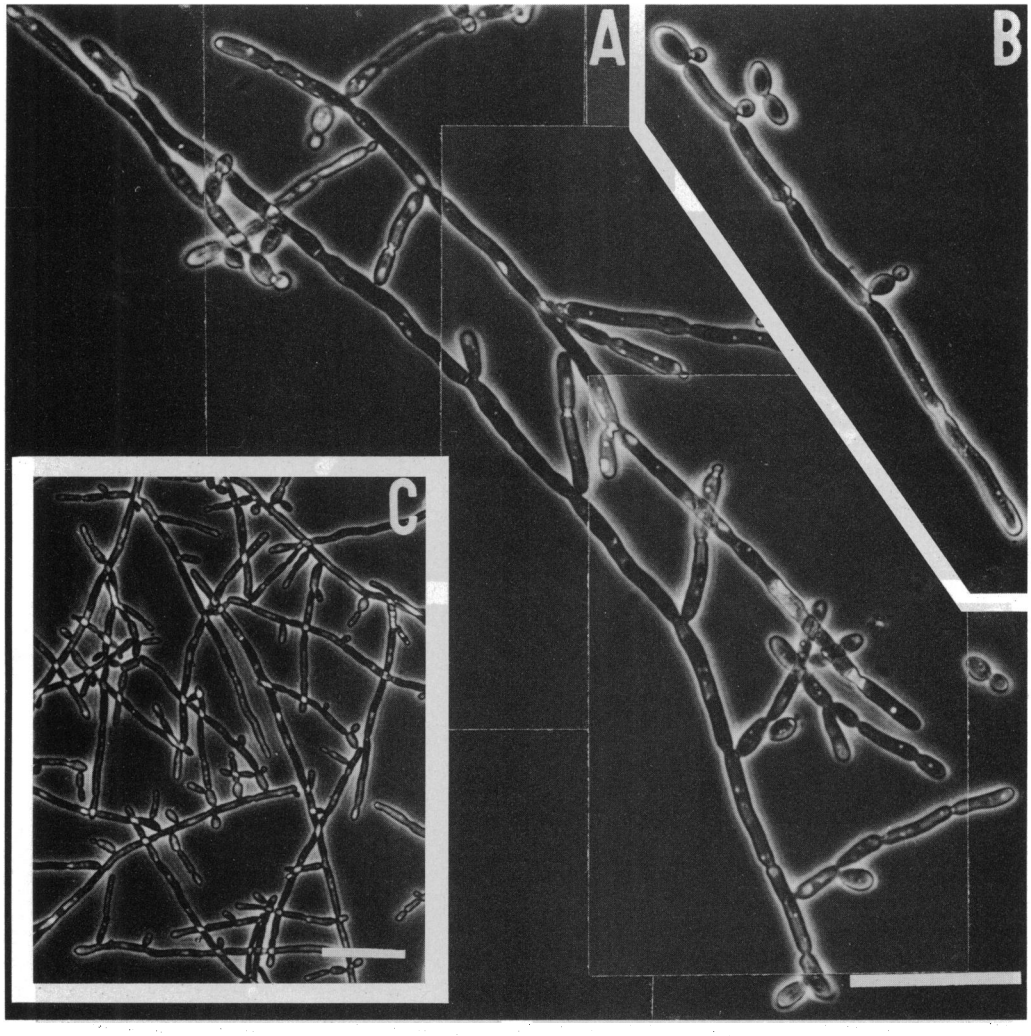


FIG. 4. Phase-contrast micrographs of long filamentous forms of *C. albicans* NEL103 grown for 24 h in YED medium. (Panel A is a composite of five microscopic fields.) Bars, 40 μm.

already displayed long filamentous structures, indicating that some of the cells had elongated in a manner that was similar to mycelial growth, with a regular formation of septa that were clearly apparent in the calcofluor-stained preparations. Hyphae branched close to septa, the branching being initiated by oval forms that eventually elongated. This pattern of growth was maintained for long periods, although the figures include only some selected samples. After 24 h (Fig. 2F and H; Fig. 3G and H; Fig. 4) the oval cells of the blastoconidial type were much more abundant, and some of them appeared to emerge from the filamentous hypha-type structures. Both mutant strains NEL102 and NEL103 displayed very similar patterns of growth in liquid cultures. Nevertheless, the filamentous structures of strain NEL103 seemed to be longer and thinner, therefore closer to hypha-type formations.

Calcofluor is known to bind preferentially to areas containing chitin (17). Budding areas, the tips of the filaments, branching areas, and septa were intensely fluorescent in the calcofluor-stained preparations, suggesting that these are the chitin-rich regions. As a comparison, we also observed preparations of the wild-type strain 1001 incubated in a medium that stimulates hypha formation. It is clear from Fig. 5 that similar structures were formed.

The cell morphologies of the two mutants described above were clearly different from those of another group of morphological mutants that we have described previously which grew permanently as filamentous and very pleomorphic structures (3). We have characterized this group as a group of pseudomycelial mutants. To initiate a genetic characterization of the strains isolated in this work, we hybridized strain NEL102 with a pseudomycelial mutant, *C. albicans* B14 ($Y^-$ $His^-$) (3), by fusing protoplasts. The complementation of nutritional deficiencies of both strains enabled us to select for prototrophic fusion products and, eventually, to obtain hybrids of these two strains. The results of this hybridization were unequivocal; prototrophic colonies of the hybrids NEL102 × B14 (not shown) were clearly smooth wild type and consisted of only oval forms of blastoconidial appearance. It follows that the genetic determinants of the altered morphologies of both strains were different and recessive in the corresponding hybrids. Sectors of rough or hairy morphology, characteristic of the parental strains,

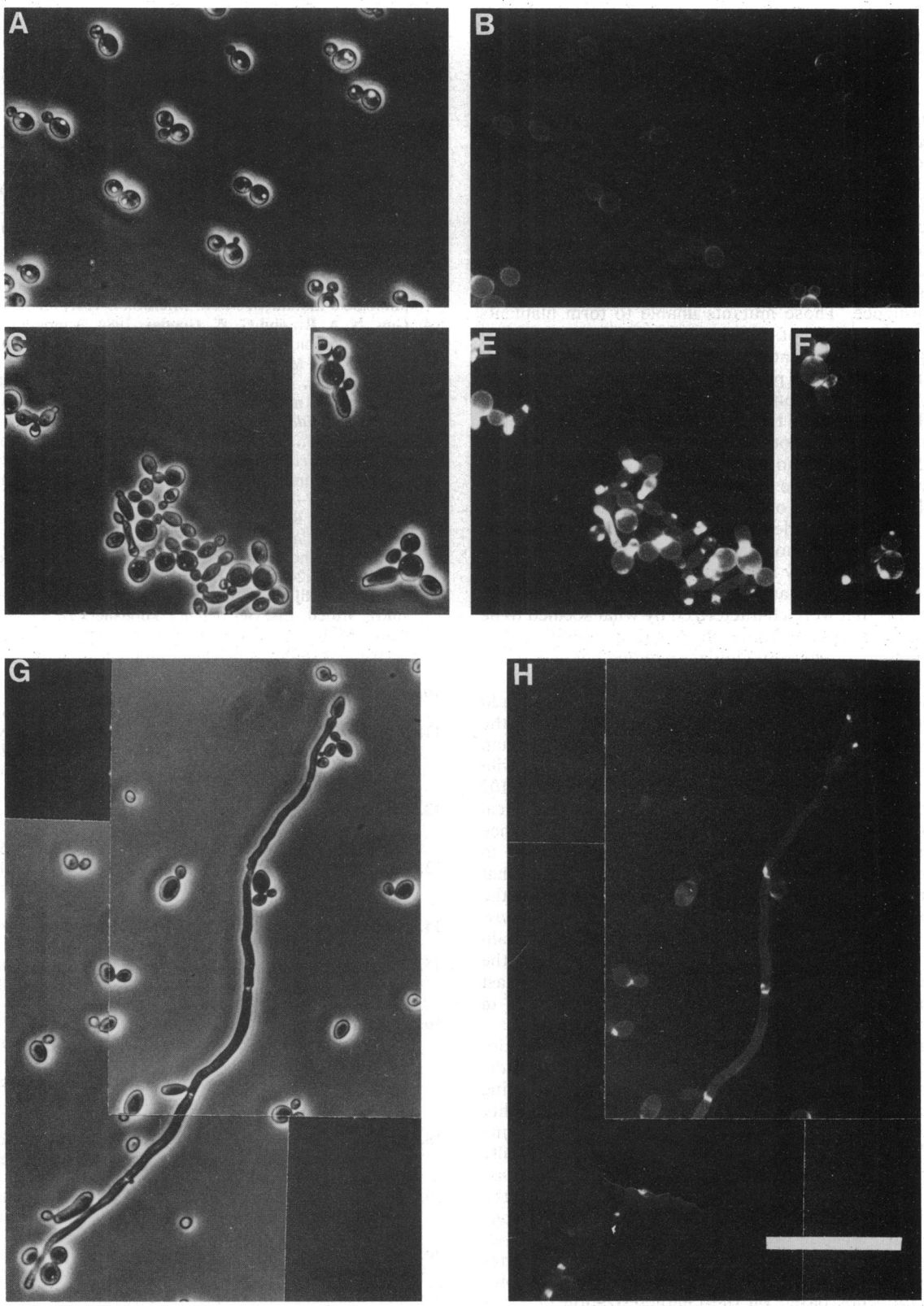


FIG. 5. Phase-contrast and calcofluor white fluorescence-stained micrographs of *C. albicans* 1001 cells incubated for different times in YE-Pro medium. (A and B) 0 h; (C, D, E, and F) 3 h; (G and H) 13 h. Bar, 40 μm. (To show the long filamentous structures, micrographs G and H are composites of two microscopic fields.)

could be observed to emerge from the smooth colonies when they were grown in complete medium after gentle UV irradiation, thus confirming the complementation of morphological deficiencies.

## DISCUSSION

Although some genetic approaches have been used to study the basis of dimorphism, the built-in controls that determine the significant changes in morphogenesis of *C. albicans* are far from elucidated. It has been clearly shown that it is feasible to isolate mutants with a phenotype that can be characterized as a morphological alteration. The capability to give rise to any type of filamentous structure (hyphae or pseudohyphae) can be lost by mutation (1, 13) without any significant alteration in viability but with the concomitant loss of virulence. Those mutants unable to form filaments represent a first class of morphological mutant. A second class might be represented by those unable to grow as blastoconidia and whose permanent morphology is similar to pseudohyphal, very pleomorphic structures (3, 13, 15). Genetic analysis has shown that mutations in at least two genes can determine this phenotype (3).

We felt that new morphological mutants closer to a permanent mycelial type were needed for a more specific genetic characterization of *C. albicans* morphogenesis. The evidence reported here clearly indicated that we were able to isolate a type of morphological mutant different from the ones reported so far. By a systematic process of mutation, mitotic recombination, and enrichment, we obtained two mutant strains that were characterized by what seemed to be the sequential expression of morphogenetic capabilities of *C. albicans*. Blastoconidia could initiate budding but eventually elongated to give rise to long, filamentous, septate, and branched structures, from which new blastoconidia could also emerge. This occurred in the absence of any of the known inducers of germ-tube formation, in nutrient medium conditions that determine only budding growth in the wild type. In fact, incubation of these mutant strains, NEL102 and NEL103, in the presence of serum, the most typical inducer of mycelium formation, did not reveal any difference in morphogenesis with regard to what was observed in nutrient medium (data not shown). It can be suggested that the mutants described here are derepressed mutants for the expression of a fundamental capability of *C. albicans*, namely, the formation of filamentous structures. Strain NEL102 complemented with B14, a representative of the second class, when they were hybridized by protoplast fusion, indicating that the genetic determinants affected in both mutants are different.

Further characterization of these mutants should contribute significantly to fundamental knowledge of basic dimorphism functions in *C. albicans*. Several strategies are being considered, but of special relevance for us is the possibility of using these strains in genetic transformation experiments to identify the genes affected by the mutations, especially when nonintegrative vectors are available for *C. albicans*. The feasibility of those vectors became clear with the cloning of DNA fragments capable of directing autonomous replication (9), and the vectors are at present being developed (M. G. Shepherd, personal communication; E. Herreros, M. Sanchez, and C. Nombela, unpublished data). Molecular approaches based on the characterization of DNA fragments capable of complementing the morphological deficiencies of these and other morphological mutants should be the correct strategy for addressing the question of specific genes involved in the control of dimorphism.

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