# *Cryptococcus neoformans STE12α* Regulates Virulence but Is Not Essential for Mating

By Y.C. Chang,* B.L. Wickes,[§] G.F. Miller,[‡] L.A. Penoyer,* and K.J. Kwon-Chung*

*From the *Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, and the ‡Veterinary Resources Program, Office of the Director, National Institutes of Health, Bethesda, Maryland 20892; and the §Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284*

## Abstract

The *Cryptococcus neoformans STE12α* gene, a homologue of *Saccharomyces cerevisiae STE12*, exists only in mating type (*MAT*)*α* cells. In *S. cerevisiae*, *STE12* was required for mating and filament formation. In *C. neoformans*, haploid fruiting on filament agar required *STE12α*. The ability to form hyphae, however, was not affected by deletion of *STE12α* when convergently growing *MATa* strains were present. Furthermore, *ste12α* disruptants were fertile when mated with *MATa* strains, albeit with reduced mating frequency. Most importantly, the virulence of a *ste12α* disruptant of serotype D strain was significantly reduced in a mouse model. When the *ste12α* locus was reconstituted with the wild-type allele by cotransformation, virulence was restored. Histopathological analysis demonstrated a reduction in capsular size of yeast cells, less severe cystic lesions, and stronger immune responses in meninges of mice infected with *ste12α* cells than those of mice infected with *STE12α* cells. Using reporter gene constructs, we found that *STE12α* controls the expression of several phenotypes known to be involved in virulence, such as capsule and melanin production. These results demonstrate a clear molecular link between mating type and virulence in *C. neoformans*.

Key words: haploid fruiting • mating assay • *STE12* • cotransformation • virulence factor

## Introduction

*Cryptococcus neoformans* is a basidiomycetous yeast pathogen that can cause life-threatening infections in normal as well as immunocompromised individuals (1). In AIDS patients, cryptococcosis is the leading cause of mycotic morbidity and mortality (2). Unlike other well studied heterothallic basidiomycetes, such as *Schizophyllum commune* and *Ustilago maydis*, which possess tetrapolar mating systems, *C. neoformans* possesses a bipolar mating system with two alleles, mating type *(MAT)α* and *MATa* (3, 4). The sexual state of *C. neoformans* is characterized by the formation of dikaryotic hyphae, which possess typical basidiomycetous clamp connections and bear terminal basidia. Repeated postmeiotic mitosis and basidiosporogenesis result in the production of four long chains of haploid basidiospores on the apical surface of the basidium. Upon germination, the basidiospores produce yeast cells that multiply by polar budding without hyphal formation. Under specific conditions, such as nitrogen starvation and dehydration, haploid yeast cells can undergo an extensive hyphal differentiation in the absence of the opposite mating type (5). Hyphae produced under these conditions possess unfused clamp connections and develop basidia bearing only *MATα*-type basidiospores. This phenomenon is called haploid or monokaryotic fruiting, and it occurs only in *MATα* strains.

The molecular analysis of hyphae production in *MATα C. neoformans* has resulted in the identification of a gene named *STE12α* whose sequence displays similarity to the *Saccharomyces cerevisiae STE12* gene (6). In *S. cerevisiae*, *STE12* is a transcriptional activator capable of inducing transcription of a number of different genes (7–11) and is well conserved among many fungi, including *Aspergillus nidulans*, *Candida albicans*, *Kluyveromyces lactis*, and *S. cerevisiae* (EMBL/GenBank/DDBJ accession nos. AF080600, U15152, L21156, and X16112, respectively). In *S. cerevisiae*, *STE12* is the target of a conserved mitogen-activated protein (MAP)[1] kinase signal transduction

Address correspondence to K.J. Kwon-Chung, LCI, NIAID, Bldg. 10, Rm. 11C304, National Institutes of Health, Bethesda, MD 20892. Phone: 301-496-1602; Fax: 301-402-1003; E-mail: June_Kwon-Chung@nih.gov

[1]*Abbreviations used in this paper:* GUS, β-glucuronidase; MAP, mitogen-activated protein; SLAD, synthetic low ammonia dextrose; YEPD, yeast extract peptone dextrose.


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pathway involved in mating, pseudohyphal development, and haploid invasive growth (for review see references 12–15). The *C. neoformans STE12α* gene, while having conserved roles in morphogenesis, has one striking difference from the *S. cerevisiae STE12* gene: it is found only in *MATα* cells (6).

It has been observed that clinical as well as environmental isolates of *C. neoformans* are predominantly *MATα* (16), and it has been shown that among serotype D strains, *MATα* cells are more virulent than *MATa* cells in the murine model (17). Genetic association of virulence and mating type has not been established among serotype A strains due to the absence of *MATa* strains. The relationship among virulence, mating type, and haploid fruiting at the molecular level is not clear. In this paper, we show that *STE12α* is important for haploid fruiting but not essential for mating. The importance of *STE12α* in regulating expression of several virulence-associated genes was also investigated. We show that *ste12αΔ* serotype D strains are markedly reduced in virulence as compared with the wild-type strain. The decreased virulence of *ste12αΔ* suggests that *STE12α* is an important regulator of virulence in serotype D and provides molecular evidence for the important role of mating type–specific gene(s) in the virulence of *C. neoformans.*

## Materials and Methods

*Strains, Media, and General Methods.* The strains used in this study were all of serotype D and are listed in Table I. B-4500 (*MATα*) is a wild-type congenic strain of B-4476 (*MATa*) previously used to investigate genetic association of mating type and virulence (17). All *C. neoformans* strains used in this study were derived from these two strains. TYCC245F1FO is a *ura5* mutant recovered from TYCC245F1 by selection on 5-fluoroorotic acid (5-FOA) medium (18). Yeast extract peptone dextrose (YEPD) contained 1% yeast extract, 2% Bacto-peptone, and 2% glucose. 3,4-dihydroxyphenylalanine (DOPA) medium was prepared as described previously (19). Minimal media contained 6.7 g of yeast nitrogen base without amino acids (Difco Labs., Inc.) with 20 g of glucose, galactose, or raffinose per liter (pH 6.0). RPMI agar (20) and V-8 juice agar (21) were prepared as described previously. Synthetic low ammonia dextrose (SLAD) medium, originally designed to induce pseudohyphal formation in *S. cerevisiae* (12), was modified by omitting histidine and using unwashed agar. Filament agar, used to induce haploid fruiting in *C. neoformans*, is similar to SLAD medium, except that filament agar contains 0.5% glucose and 4% agar (5). Culture conditions for RPMI growth were 30°C with 10% $CO_2$ (20). All crosses were performed on V-8 juice agar as described previously (21). Phospholipase activity was determined on egg yolk agar as described (22). In brief, the egg yolk agar contained Sabouraud dextrose agar with 1 M sodium chloride, 0.005 M calcium chloride, and 8% sterile egg yolk. Each isolate was tested in triplicate. Cultures were incubated at 30°C for 72 h, and the diameter of the zone of precipitate around the colonies was measured. The ratio of the diameter of the colony to the total diameter of the colony plus precipitation was measured as an index of phospholipase activity. There is an inverse relationship between index and enzyme activity: the smaller the index, the higher the enzyme activity of the strain.

*Plasmids.* Plasmids used in this study are listed in Table II. Plasmid pYCC245 was the deletion construct (see Fig. 1) in which the DraIII/BamHI fragment of *STE12α* in p18-S1 (6) was replaced with the 3.0-kb BamHI/EcoRI fragment of the *ADE2* gene from pYCC76 (23). The *URA5* gene was placed at the 5′ flanking region of *STE12α* in pYCC245. Plasmid pYCC259 was constructed as follows. An NdeI site was created at the first ATG site of the coding region of *STE12α* by PCR, and the resulting construct was placed under the control of the *GAL7* promoter as described (24). Plasmid pYCC328 contained a promoterless β-glucuronidase (*GUS*) gene and the 1.08-kb stability factor (STAB) sequence (25). STAB confers an autonomous replication sequence–like function, which enhances the episomic maintenance of plasmids in *C. neoformans.* Incorporation of the STAB sequence in our GUS reporter constructs greatly reduced the variation of the assay results compared with constructs without STAB (our unpublished results). For *GUS* reporter gene constructs, an NdeI site was created at the first ATG site of the coding region of each gene by PCR. Individual promoters were then cloned into the NdeI site created at the first ATG of the *GUS* gene. Sequences of all PCR clones were confirmed before use.

*GUS Activity Assay.* B4500F0, TYCC259, and TYCC245F1FO were transformed with various GUS reporter constructs (Table II). Six independent transformants from each construct were assayed for GUS activity. For overexpression of *STE12α*, cells

**Table I.** *List of Strains Relevant to this Study*

| Strain | Genotype/comment | Source |
|---|---|---|
| B4500 | *MATα*; congenic strain of B-4476 | Reference no. 17 |
| B4476 | *MATa*; congenic strain of B-4500 | Reference no. 17 |
| B4500FO2 | *MATα ura5* | Reference no. 23 |
| LP1 | *MATα ura5 ade2* | Reference no. 23 |
| JEC30 | *MATa lys1* | Gift of J.C. Edmen |
| JEC32 | *MATa lys2* | Gift of J.C. Edmen |
| TYCC259 | MAT*α ura5 ade2 GAL7(p)::STE12α::ADE2* | This study |
| TYCC245 | *MATα ura5 ade2 Δste12a::ADE2*; derived from LP1 | This study |
| TYCC245F1 | *MATα Δste12::ADE2*; $F_1$ of TYCC245 × JEC32 | This study |
| TYCC245F1FO | *MATα ura5 Δste12::ADE2*; derived from TYCC245F1 | This study |
| TYCC409A | *MATα ura5 ade2*; *STE12α* reconstituted | This study |
| TYCC409AF1 | *MATα*; $F_1$ of TYCC409A × JEC30 | This study |

**Table II.** *List of Plasmids Relevant to This Study*

| Plasmid | Gene/description | Source |
|---|---|---|
| pCIP3 | *URA5* | Reference no. 26 |
| pNH7 | *STE12α URA5* | Reference no. 26 |
| p18-S1 | SphI/EcoRI subclone of *STE12α* | Reference no. 26 |
| pYCC76 | *ADE2* | Reference no. 23 |
| pYCC245 | *STE12α* deletion construct; see Fig. 1 | This study |
| pYCC259 | *GAL7(p)::STE12α* in *ADE2* plasmid | This study |
| pYCC318 | *CAP59(p)::GUS* in pYCC328 backbone | This study |
| pYCC319 | *CAP64(p)::GUS* in pYCC328 backbone | This study |
| pYCC320 | *CNLAC1(p)::GUS* in pYCC328 backbone | This study |
| pYCC325 | *CAP60(p)::GUS* in pYCC328 backbone | This study |
| pYCC327 | *ADE2(p)::GUS* in pYCC328 backbone | This study |
| pYCC328 | 1.1-kb STAB in pCIP3 with promoterless *GUS* | This study |
| pPM8 | STAB, *URA5*, telomeres | Gift of P. Mondon |

were inoculated in 10 ml of minimal medium containing 2% raffinose in a 50-ml Falcon tube and incubated at 30°C with shaking at 200 rpm for 24 h. 1 ml of the culture was inoculated into 9 ml of fresh minimal medium containing either 2% glucose or 2% galactose as a carbon source, and the culture was incubated for an additional 20 h. Cells were harvested, and GUS activity was assayed as previously described (24). Activity was expressed as picomoles of 4-methylumbelliferone produced per minute per 200 μg of protein. To assay GUS activity of cultures in late stationary phase, cells were grown in 10 ml of minimal medium containing 2% raffinose for 24 h, and 1 ml of this culture was inoculated into 9 ml of fresh minimal medium containing 2% glucose. This culture was then grown for an additional 45 h under the same conditions. Cells were harvested, and GUS activity was assayed.

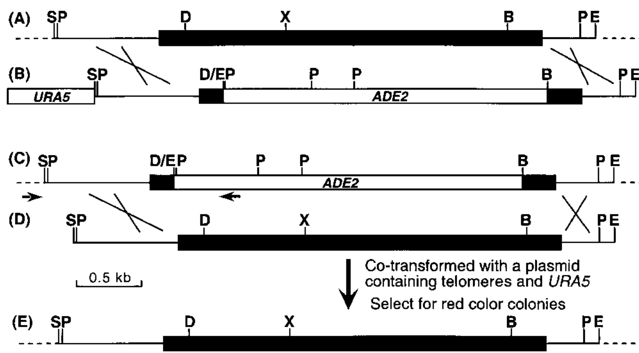
*Transformation of C. neoformans and Disruption of STE12α.* The electroporation method described by Edman and Kwon-Chung (26) was used to transform *C. neoformans*. To disrupt *STE12α*, the positive–negative selection protocol was used as described previously (20). To identify putative *ste12α* disruptants, ~500 transformants that grew on 5-FOA plates after transformation were inoculated into 100 μl of water as pools of 10. An aliquot of each pool was then added to 50 μl of 1× PCR reaction buffer, heated at 95°C for 15 min, and processed for PCR. From pools that gave positive reactions, another round of PCR was performed on individual clones to identify the true positive clones. With this method, we identified two *ste12α* disruptants that were confirmed by Southern analysis.

*Cotransformation Method.* A telomere-based plasmid containing *URA5* and STAB (pPM8; a gift from P. Mondon, NIH, Bethesda, MD) was linearized to expose the telomere ends. The linearized DNA was mixed with the 4.5-kb SphI/EcoRI fragment of p18-S1, which contained *STE12α*. To deliver the DNA into TYCC245, both electroporation and biolistic transformation (27) were tested. After transformation, the cells were plated on minimal medium supplemented with adenine and incubated at 30°C for 1 wk, and colonies showing a red color were isolated. All putative clones were transferred on YEPD three times to cure the telomere-based plasmid. Uracil and adenine auxotrophs were isolated, and DNAs were analyzed by Southern blot. For the electroporation method, two red colonies were isolated from ~$10^5$ transformants, and both clones displayed a Southern pattern of wild-type *STE12α*. For the biolistic method, five red colonies were isolated from ~7,000 transformants, and four of them showed Southern patterns identical to that of wild-type *STE12α*.

*Quantitative Assay for Mating Frequency.* The rationale is similar to the quantitative mating assay used in *S. cerevisiae* (28). All cultures used in the mating assay were <24 h old. About $2 \times 10^6$ viable cells from two opposite mating type strains, each carrying different auxotrophic markers, were mixed in 200 μl of 0.9% NaCl in a microfuge tube. The cells were collected on a 0.45-μm filter by suction, and the filters were placed on V-8 agar plates for 7 h at room temperature. Cells were then washed off the filter and spread on minimal media plates, which were incubated for 4 d at 30°C. The number of colonies producing hyphae was then determined. None of the cells from each mating parent would grow on minimal media, because each mating parent carried a different auxotrophic marker. The mating frequency of each strain was determined as the total number of hyphae producing colonies/total number of input cells from both mating type strains. The relative mating frequency was expressed as a percentage of the mating frequency of the wild-type reference strain (LP1). Two different auxotrophic *MATa* strains (JEC30 and JEC32) were used as tester strains to determine the mating frequency of any given *MATα* strain. Data were the average of results derived from mating with JEC30 and JEC32. The experiments were repeated at least twice to confirm reproducibility.

*Virulence Study.* Female BALB/c mice (6–8 wk old) were injected via the lateral tail vein with 0.2 ml of a suspension of each yeast strain and mortality was monitored. Kaplan-Meier analysis of survival was performed using JMP software for the Macintosh (SAS Institute). To measure the growth rate of each strain in the brain, mice were injected with yeast cells ($3 \times 10^5$ cells) as above, and then three mice each were killed at several intervals after injection (3 h as the starting point and 3, 6, 9, 12, and 15 d after injection). The brains were homogenized with a mortar and pestle, diluted, and then plated onto YEPD agar plates. Colonies were counted after incubation at 30°C for 2 d.

*Histopathology.* Brains were removed and fixed in 10% buffered (neutral) formalin. Paraffin sections of the brains were stained with hematoxylin and eosin and Gomori methenamine silver (performed by American Histolabs).



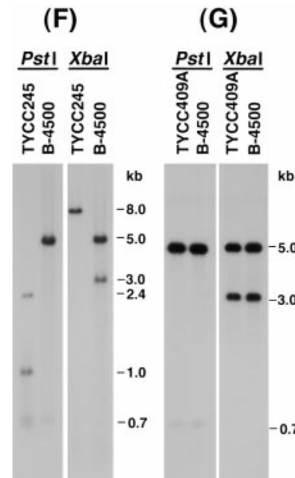




**Figure 1.** (A) Map of the *STE12α* gene. (B) Map of *STE12α* deletion construct. For simplicity, the *URA5* gene is not drawn to scale. (C) Map of *ste12α* in TYCC245. (D) Map of DNA fragment used in cotransformation. (E) Map of reconstituted *STE12α*. Arrowheads, primer locations. Black boxes, coding region of *STE12α*. Crosses, crossing over. Dashed lines, chromosomal region flanking *STE12α*. B = BamHI; D = DraIII; E = EcoRI; P = PstI; S = SphI; X = XbaI. (F) Southern blot of *ste12α* deletant. (G) Southern blot of *STE12α* reconstituted strain. B-4500, wild type; TYCC245, *ste12α* deletant; TYCC409A, *STE12α* reconstituted strain. DNA was isolated, digested with restriction enzymes, fractionated on 0.8% agarose gel, and analyzed by Southern blot. The blots were hybridized with a probe of the SphI/EcoRI fragment of *STE12α*.

## Results

*Disruption of STE12α.* To understand the function of *STE12α* in serotype D strains, we deleted the *STE12α* gene from LP1 by a positive–negative selection method (20). With this method, the DraIII/BamHI region, which contains the majority of the *STE12α* coding region, was replaced by the *ADE2* gene in *ste12α* disruptants by homologous integration (Fig. 1, A–C). Because *ste12α* disruptants would not be expected to have a visible phenotype, we first screened all transformants by PCR using primers designed to detect clones in which *STE12α* had been deleted (Fig. 1 C, arrowheads). Only clones containing a homologous integration of the deletion construct would yield the predicted PCR product. PCR positive clones were then analyzed by Southern blot to confirm a true gene replacement event. The Southern blot pattern in Fig. 1 F demonstrated that the signals of the wild-type *STE12α* band in B-4500 were replaced by new signals in one of the putative *ste12α* disruptants, TYCC245. Therefore, TYCC245 contained a *ste12α* disruption.

*STE12α and Virulence-associated Genes.* As the *STE12α* gene is *MATα* strain specific and *MATα* is known to be more virulent than *MATa* strains, it was of interest to determine if deletion of *STE12α* would change the expression levels of known virulence-associated genes. The laccase gene (*CNLAC1*) and three capsule-associated genes (*CAP59*, *CAP60*, and *CAP64*) have been identified as virulence factors in *C. neoformans* (20, 23, 29, 30). The promoters of these genes were individually fused with the *GUS* gene in a *URA5*-containing plasmid (Table II). *GUS* reporter constructs of the four virulence-associated genes were transformed into TYCC245F1FO (*ste12α::ADE2*, *ura5*) as well as a wild-type control strain (B-4500FO2), and GUS activity was determined. GUS activity in transformants of B-4500FO2 containing constructs of *CAP59*, *CAP60*, *CAP64*, and *CNLAC1* was generally much lower in cells taken from glucose-grown overnight cultures (20-h cultures; Table III, column 1) compared with cells from late stationary phase glucose-grown cultures (45-h cultures; Fig. 2, black bars). It has been documented that cells in late stationary phase express higher laccase activity (19) and larger capsules compared with cells in the exponential growth phase (31). Therefore, the observations that cells in

**Table III.** *Effect of Overexpression of STE12α on GUS Activity*

| | B4500FO2 | | TYCC259 | |
|---|---|---|---|---|
| | Glucose | Galactose | Glucose | Galactose |
| *CAP59* | 2.30 ± 1.27 | 3.63 ± 1.11 | 3.00 ± 1.70 | 21.07 ± 4.09 |
| *CAP64* | 0.30 ± 0.21 | 2.10 ± 0.30 | 0.53 ± 0.30 | 13.57 ± 2.48 |
| *CAP60* | 5.52 ± 2.66 | 6.88 ± 5.05 | 2.73 ± 2.41 | 14.13 ± 4.57 |
| *CNLAC1* | 1.92 ± 1.38 | 2.23 ± 1.58 | 1.96 ± 0.62 | 18.97 ± 5.41 |
| *ADE2* | 0.20 ± 0.18 | 1.77 ± 0.63 | 0.43 ± 0.39 | 7.92 ± 2.42 |
| Vector | 0.17 ± 0.15 | 1.13 ± 0.45 | 0.40 ± 0.53 | 5.87 ± 0.97 |

the stationary phase displayed higher reporter activity corroborated these reports. When GUS activity of late stationary phase cultures was compared in the *ste12α* or wild-type background, it was clear that all four constructs produced lower GUS activity in the *ste12α* background (Fig. 2). In contrast, GUS activity was very low and showed no difference in transformants with constructs containing the *ADE2* promoter or in a vector with a promoterless *GUS* gene (Fig. 2). Reductions in capsule and melanin production, however, were not apparent by visual inspection when the *ste12α* strain was grown on agar media (YEPD or RPMI for capsule, DOPA for melanin; data not shown).

As the *ste12α* disruptant influenced the expression levels of four virulence-associated genes, we further investigated the effect of overexpression of the *STE12α* gene. *STE12α* was placed under control of the *C. neoformans GAL7* promoter in an *ADE2*-containing plasmid. The resulting plasmid was transformed into strain LP1 (*MATα, ade2, ura5*), and a stable transformant (TYCC259) was obtained (Table I). PCR with construct-specific primers confirmed that the *GAL7(p):: STE12α* fusion was intact in TYCC259. When TYCC259 was grown on minimal media containing galactose as the sole carbon source, it produced hyphal projections similar to the phenomenon observed by Wickes et al. (6), which was interpreted as being a result of overexpression of *STE12α*. *GUS* reporter constructs of four virulence-associated genes were transformed into TYCC259 as well as a wild-type control strain (B-4500FO2), and GUS activity was determined. GUS activity was found to be relatively low in transformants of B-4500FO2 when the cells were grown in either glucose or galactose (Table III). Similarly low levels of GUS activity were detected in transformants of TYCC259 when glucose was used as the sole carbon source. When transformants of TYCC259 were grown in galactose, however, GUS activity increased significantly (Table III). The increase in GUS activity was most dramatic in transformants containing a fusion of *CAP64*. Although GUS activity also increased in transformants containing constructs with the promoter of *ADE2* or in the vector with a promoterless *GUS* gene, the increase was not as large in the B-4500FO2 background (TYCC259 versus B-4500FO2). Although the reason is not clear, it may be that some sequences in these constructs respond directly or indirectly to the overexpression of *STE12α*. Therefore, although the influence of overexpression of *STE12α* on the virulence-associated genes was not as dramatic as that of the *ste12α* disruptant, it was clear that overexpression of *STE12α* enhanced the *CAP64* reporter activity.

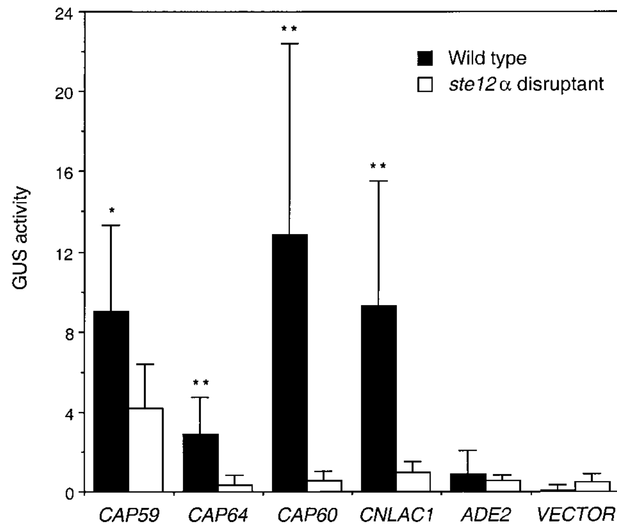



**Figure 2.** GUS activity assay for stationary phase cells. The *GUS* reporter constructs were transformed into wild type (B-4500FO; black bars) and *ste12α* disruptant (TYCC245F1FO; white bars). The cells from stationary phase were used for GUS activity assay. Six independent transformants from each construct were assayed for GUS reporter activity. Error bars represent the sample SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

As phospholipase was suggested to be a putative virulence factor for *C. neoformans* (22), we tested the effect of deletion of *ste12α* on phospholipase activity. The activity of phospholipase on egg yolk agar was considerably higher in the control strain (B-4500) than that in the *ste12α* disruptant (TYCC245F1). The index of phospholipase activity of B-4500 was one-half of that produced by TYCC245F1 ($0.54 \pm 0.04$ versus $0.97 \pm 0.04$). Thus, deletion of *ste12α* downregulated the activity of extracellular phospholipase.

*STE12α and Mating.* In *S. cerevisiae*, *STE12* is required for mating and filament formation, as *ste12* mutants are sterile and do not produce pseudohyphae (8, 15, 32). We first tested if mating ability was altered in *C. neoformans* when *STE12α* was deleted. TYCC245 (*ste12αΔ*) was crossed with a *MATa* strain (B-4476) on V-8 juice agar. We observed that the mating produced hyphae with fused clamp connections and viable basidiospores, as seen in the wild-type *MATα* × *MATa* crosses (control). Thus, surprisingly, TYCC245 was fertile. When the progeny of the TYCC245 × *MATa* cross were analyzed, a predicted segregation pattern of genetic markers was observed, confirming that *ste12α* mutants were fertile (data not shown). The number of basidia with healthy spore chains, however, was reduced compared with the control. To assess the extent of reduction in mating, mating frequency was determined quantitatively and found to be reduced almost 93% in *ste12α* strains (Fig. 3). These results indicated that although the mating frequency of the *ste12α* disruptant was greatly reduced, deletion of *ste12α* did not abolish sexual reproduction.

*STE12α and Haploid Fruiting.* In *S. cerevisiae*, both pseudohyphal formation and haploid invasive growth require the presence of a functional *STE12* gene (13, 33). Filamentous growth, however, is only partially defective in *C. albicans* when both copies of *CPH1*, a *STE12* homologue, are disrupted (32). We tested whether *MATα* strains can undergo haploid fruiting in the *ste12α* background. We found that the *ste12α* deletant failed to produce haploid fruiting when it was placed on filament agar. In contrast, the wild-type control strain produced abundant hyphae and possessed unfused clamp connections and spore chains, typical features of haploid fruiting (Fig. 4). These results suggested that while *ste12α* disruptants were still fertile, they were incapable of undergoing haploid fruiting on filament agar.

*STE12α and Filament Formation.* We have previously

**Figure 3.** Quantitative assay for mating frequency. Cells from two opposite mating type strains each carrying different auxotrophic markers were placed on filters and incubated on V-8 agar plates. Cells were washed off the filters and plated on minimal media. Photograph shows colonies producing abundant hyphae as a result of mating. The background cells were the input cells that failed to complement the nutritional deficiency in each mating type.

observed that *MAT*$\alpha$ cells produce hyphae within 24 h when streaked in close proximity (<500 $\mu$m) with *MATa* cells on SLAD medium (our unpublished observation). SLAD medium has a composition similar to filament agar and has been shown to induce pseudohyphal formation in *S. cerevisiae* (12). When *MAT*$\alpha$ cells of *C. neoformans* were grown alone on SLAD medium, no hyphae were produced. It was of interest to investigate whether *ste12*$\alpha$ disruptants lost the ability to produce hyphae on SLAD medium in response to the presence of *MATa* cells. Fig. 5 A shows hyphal production by B-4500 (*STE12*$\alpha$, *MAT*$\alpha$) when streaked in parallel to a streak of B-4476 (*MATa*) within a 500-$\mu$m distance, whereas *MATa* cells failed to respond in a similar manner (no hyphal filaments were produced). Similarly, no hyphal structures were detected when two streaks of *ste12*$\alpha$ (TYCC245F1) cells were paired on SLAD agar (Fig. 5 B). TYCC245F1 produced hyphae only when it was streaked in parallel with *MATa* cells in close proximity (<500 $\mu$m distance; Fig. 5 C). The hyphae produced by TYCC245F1 (Fig. 5 C), however, were morphologically different, tending to be more sinuous compared with hyphae produced by B-4500 (Fig. 5 A). When plates containing parallel streaks of opposite mating type strains were incubated for 1 wk, hyphae produced by the *MAT*$\alpha$ strain reached *MATa* cells, and both basidia and spore chains were produced sporadically at the margin of *MAT*$\alpha$ streaks (data not shown). Similar results were observed on SLAD agar without ammonium sulfate, as well as on minimal medium (SLAD agar with 37.8 mM ammonium sulfate). It is clear, therefore, that there are at least two different signaling pathways leading to hyphal formation in *C. neoformans.* These observations suggest that although the *ste12*$\alpha$ disruptant lost its ability to undergo haploid fruiting in response to nitrogen starvation, it retained the ability to form hyphae in response to the presence of *MATa* cells on SLAD agar.

*Virulence Studies.* Because the GUS data suggested that

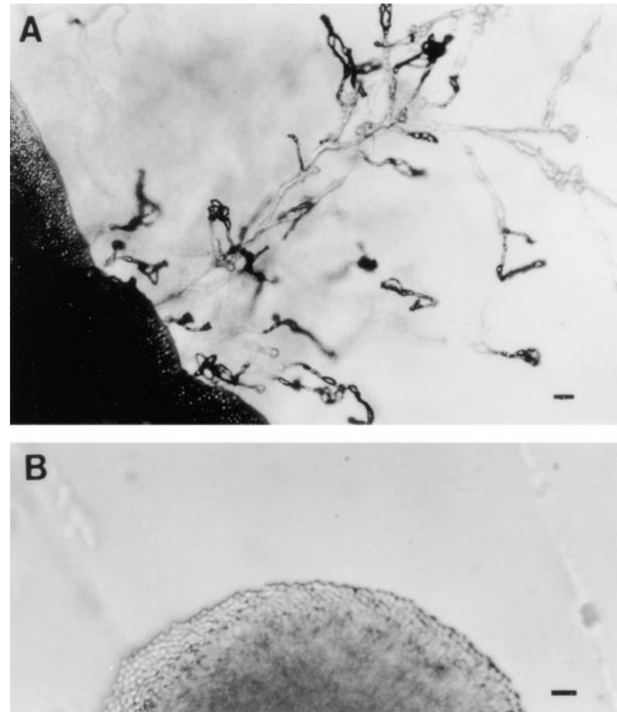



**Figure 4.** Haploid fruiting. Cells from wild-type B-4500 (A) and TYCC245F1 (B) were inoculated on filament agar and incubated at room temperature for 5 d (bar = 15 $\mu$m). Only the wild-type strain produced abundant hyphae and basidia with spore chains.

*STE12*$\alpha$ has an effect on the expression of some of the virulence-associated genes under certain growth conditions, it was of interest to test the role of *STE12*$\alpha$ in virulence using an animal model. Groups of mice were infected with an $F_1$ prototroph of the *ste12*$\alpha$ disruptant (TYCC245F1) and the wild-type congenic strain (B-4500). The *ste12*$\alpha$ disruptant produced significantly lower mortality than the wild-type (*STE12*$\alpha$) strain, and the average number of days of survival was much longer in mice infected with the *ste12*$\alpha$ disruptant (log-rank, $P < 0.0001$; Fig. 6 A). When the growth rate of TYCC245F1 and B-4500 was compared at 37°C in YEPD broth, no obvious difference was observed. To test if the growth rates of these two strains were different in vivo, groups of mice were injected and the fungal burden in the brain was determined at several intervals up to 15 d after infection. Interestingly, similar numbers of CFUs were observed in the brains of mice infected with TYCC245F1 and B-4500, again suggesting no difference in growth rate (data not shown). However, the capsule size of yeast cells in brain smears from mice infected with TYCC245F1 was smaller (ranging from 0.5 to 3 $\mu$m) than seen in mice infected with B-4500 (ranging from 1 to 7 $\mu$m; Fig. 7). The histopathology of the mouse brains showed that the neuronal parenchyma in both groups of mice was disrupted to a similar degree by large multiloculated lesions that contained both yeast cells and large, foamy macrophages (Fig. 8, B and D). However, mice infected with TYCC245F1 had a greater number of inflammatory cells (lymphocytes, macrophages, and a few neutro-

Figure 5. Hyphal formation on SLAD medium. (A) B-4500 × B-4476. (B) TYCC245F1 × TYCC245F1. (C) TYCC245F1 × B-4476. Cells of each isolate were streaked in parallel on SLAD medium and incubated at room temperature for 24 h (bar = 15 μm).

phils) surrounding vessels and in meninges (Fig. 8 C) than those infected with the wild-type strain (Fig. 8 A). In addition to being infiltrated by fewer inflammatory cells, the meninges of mice infected with the wild-type strains were more severely expanded by clear cystic spaces and yeast cells (Fig. 8 A) than were those of mice infected with TYCC245F1 (Fig. 8 C). These observations suggested that the *ste12α* strain provokes a stronger immune response than the *STE12α* strain. When viewed in light of the GUS experiments, these data implicate the importance of *STE12α* in regulating the expression of some of the known virulence-associated genes in vivo.

*Reconstitution of STE12α.* To test if reintroduction of the wild-type *STE12α* gene into the *ste12α* disruptant would allow the fungus to regain its virulence, TYCC245F1FO was separately transformed with two *URA5*-containing plasmids, the vector (pCIP3) and a pCIP3-based plasmid containing wild-type *STE12α* (pNH7). Stable transformants were selected after repeated transfer on YEPD agar. Because *C. neoformans* often modifies incoming DNA after electroporation, PCR was used to identify integrative transformants containing an intact *STE12α* gene. Strains containing an intact *STE12α* gene were able to produce wild-type hyphae when analyzed by the SLAD assay in the presence of a *MATa* strain nearby (data not shown). In contrast, transformants that received only the vector (pCIP3) produced sinuous hyphae indistinguishable from hyphae produced by the *ste12α* disruptant. Considering the fact that transformed DNA tends to integrate randomly at ectopic sites in *C. neoformans*, three independent stable transformants, each derived from the pCIP3 and pNH7 constructs, were chosen for virulence studies. Two of the three transformants of pNH7 produced mortality earlier, with the total number of mice surviving at the end of the experiment considerably less than that of all the other transformants. One pNH7 transformant, however, produced mortality indiscernible from the transformants of pCIP3. When the results from pNH7 and pCIP3 were pooled and the difference in survival among these two groups was compared, there was a significant difference between mice receiving pNH7 and pCIP3 (log-rank, $P = 0.007$).

It is possible that an ectopic integration could alter ex-

Figure 6. Virulence studies. BALB/c mice were challenged with (A) $5 \times 10^5$ yeast cells or (B) $10^6$ yeast cells via the lateral tail vein. The mortality was monitored after the injection. B-4500, wild type; TYCC245F1, *ste12α* disruptant; TYCC409AF1, *STE12α* reconstituted strain.

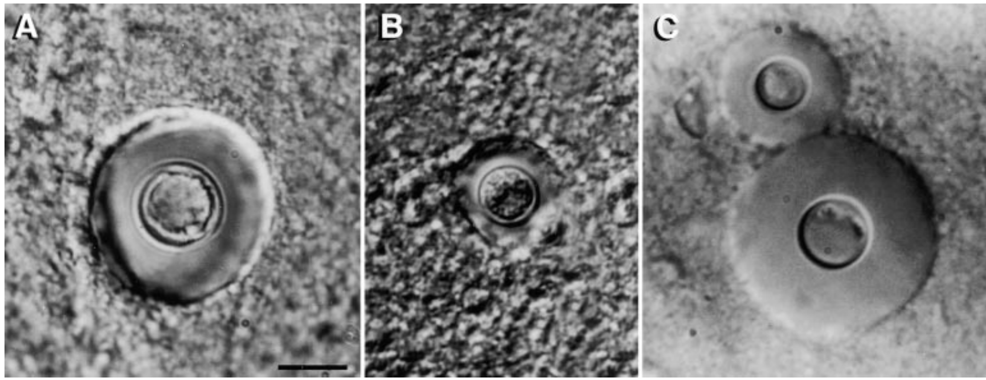



**Figure 7.** Brain smear showing cells of B-4500 (A), TYCC245F1 (B), and TYCC409AF1 (C). Brain tissue of mice challenged with different yeast strains was smeared on a microscopic slide and examined under a microscope with a Normalski interference condenser. Yeast cells with the largest capsule observed in the smears were photographed (bar = 10 μm).

pression, either through positional effects or subtle modifications of the construct, which could lead to unexpected phenotypes. As only two out of the three pNH7 transformants restored virulence, it was necessary to address this variable. This was done by reconstituting the deleted *ste12α* locus back to the wild type by cotransformation. A DNA fragment containing *STE12α* and a telomere-based *URA5* plasmid were mixed and transformed into TYCC245 (Fig. 1, C–E). Because telomere-based plasmids have a very high frequency of transformation and can be easily cured due to their instability (34), one such plasmid was used as a carrier of the *URA5* gene in the cotransformation. The resulting transformants were selected on minimal medium supplemented with adenine. If the DNA fragment containing *STE12α* integrated into the genome homologously and replaced the deleted *ste12α* gene by double cross-over, the resulting transformants would be auxotrophic for adenine and produce red-colored colonies on minimal medium supplemented with a suboptimal level of adenine. Red-colored transformants were indeed observed. These transformants were isolated and transferred to YEPD medium to cure the cotransformed *URA5* plasmid. Fig. 1 G is the Southern blot analysis of one of the transformants (TYCC409A) showing that the hybridization patterns of the wild-type *STE12α* gene were restored in this strain.

TYCC409AF1, a prototrophic progeny of TYCC409A crossed with JEC30 (*MATa*, *lys1*), produced hyphae on filament agar and exhibited a hyphal morphology similar to B-4500 on SLAD medium (data not shown). The mating frequency of TYCC409A was 86.6% of that of LP1. The phospholipase activity of TYY409AF1 was close to the wild-type B-4500 (0.61 ± 0.05 versus 0.50 ± 0.04). Therefore, the in vitro wild-type phenotypes were almost completely restored in the *STE12α*-reconstituted strains.

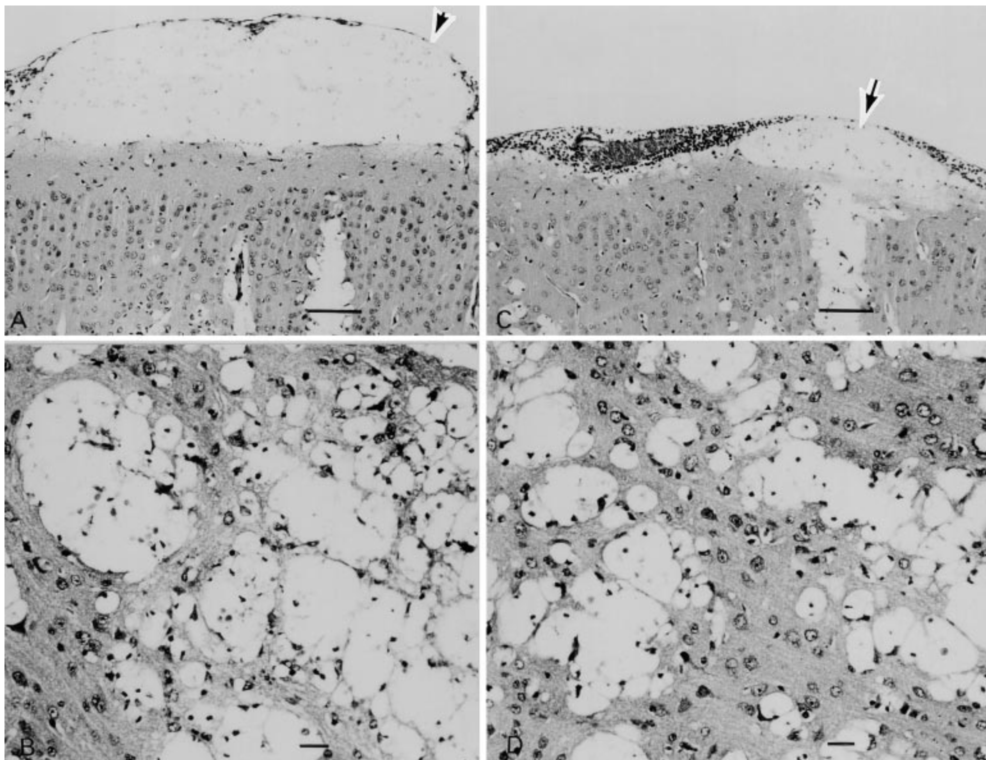



**Figure 8.** Histopathology of mice infected with wild type (A and B) and *ste12α* disruptant (C and D) (hematoxylin and eosin staining). (A). The meningeal area showing large cystic space (arrow) containing Cryptococci (light dots) with few immune cells (dark dots) (bar = 100 μm). (B). Neural parenchyma showing extensive formation of locular lesions containing yeast cells (bar = 50 μm). (C) The meningeal area showing smaller cystic space (arrow) and a large number of immune cells (bar = 100 μm). (D). Neural parenchyma with extensive locular lesions containing yeast cells (bar = 50 μm).

The virulence of the *STE12α*-reconstituted strain was compared with that of the wild-type congenic strain, B-4500. Fig. 6 B shows that the virulence was nearly restored when the wild-type *STE12α* gene was reintroduced into the genome of the *ste12α* disruptant. Furthermore, the capsule size of yeast cells in brain smears from mice infected with the *STE12α*-reconstituted strain was the same (1–7 μm) as that of mice infected with B-4500 (Fig. 7 C). Therefore, these observations fulfill the molecular version of Koch's postulates for *STE12α* as a factor associated with virulence (35).

## Discussion

The *S. cerevisiae STE12* gene is a key component of two MAP kinase cascades involved in mating and filamentous/invasive growth (12–15). Although *STE12α* shows sequence similarity with *STE12* of *S. cerevisiae*, single copy or overexpression of *STE12α* cDNA in *S. cerevisiae* failed to complement the *ste12* phenotypes, including the ability to mate and form pseudohyphae (data not shown). Recent identification of another *C. neoformans MATα*-specific gene, *STE11α*, which belongs to the same cascade, indicates an unusual arrangement of the MAP kinase cascade in *C. neoformans* (6). One of the intriguing findings in our study is that in contrast to the *S. cerevisiae ste12* mutants, the *C. neoformans ste12α* disruptant was still able to mate, albeit with reduced frequency. The fact that *STE12α* is dispensable for mating further demonstrates the uniqueness of the mating pathway of *C. neoformans*.

Mating of *C. neoformans* is usually performed on V-8 juice agar and requires physical contact of cells from both mating types. In general, it is difficult to measure mating frequency accurately in *C. neoformans* (36). In our assay, both the *ste12α* deletant and the reconstituted strain carried auxotrophic markers. The *MATa* tester strains carried a different nutritional marker to allow the discrimination of mated and unmated cells, as unmated cells could not grow on minimal media. Only cells of the opposite mating type could fuse and produce viable colonies that, in turn, could be detected visually. Thus, our assay measured how frequently cells of opposite mating type fused and formed colonies by compensating for the nutritional deficiency in each individual mating type. This process appeared to require several hours to develop, as no colony grew on minimal medium when the filters containing cells of both mating types were incubated for $<2$ h (data not shown). We noticed that most of the colonies grew on minimal media as hyphal tufts. This phenotype is typical of heterokaryons formed by fusion of two opposite mating types and has been reported previously (37). This morphology is distinct from the analogous assay in *S. cerevisiae* in which the diploid grows as yeast. In general, the mating frequency for the wild-type (LP1) ranged from $10^{-3}$ to $10^{-4}$ of input cells. Deletion of *ste12α* reduced the mating frequency to 6.7% of LP1, whereas the mating frequency of the reconstituted strain was 86.6% of LP1. Although the 10–15-fold reduction in mating efficiency for *ste12α* mutants appears substantial, the degree of reduction is negligible compared with *S. cerevisiae ste12* mutants (38). It is possible that *C. neoformans* contains another *STE12* homologue that could substitute for the function of *STE12α* protein during the mating process and would explain the minor effect of *ste12α* on mating. This possibility has prompted us to further explore the *C. neoformans* genome. We have tentatively identified a *STE12* homologue in *MATa* strains of *C. neoformans* (our unpublished results). If this *STE12* homologue proves to be functional, *C. neoformans* would be the first reported species containing two different *STE12* homologues in two opposite mating type strains, and this discovery would further portray the uniqueness of the mating pathway in *C. neoformans*.

One of the intriguing findings in this study was that SLAD medium, independent of the presence of ammonium sulfate, supports hyphal formation in *MATα* strains if a *MATa* strain is in close proximity. Induction of hyphal formation occurs on SLAD medium without contact of the two opposite mating types and only occurs in *MATα* cells. In the SLAD assay, it is reasonable to assume that pheromones or other secreted compounds from *MATa* strains induce hyphal formation in *MATα* strains. However, these hyphal strands should not be viewed as typical conjugation tubes produced in response to the opposite mating type. *C. neoformans* cells do not reach out to cells of the opposite mating type by producing filamentous conjugation tubes (39). *C. neoformans* cells produce very short conjugation tubes only upon contact with cells of the opposite mating type. This phenomenon is different from many other heterothallic basidiomycetous yeasts, which produce conjugation tubes that resemble germlings in response to the opposite mating type before fusion (40, 41). Recent isolation of a gene encoding the pheromone in *MATa* strains (Wickes, B.L., unpublished results) will allow us to test if hyphal formation can be induced by synthetic *a*-pheromone in single *MATα* cultures growing on SLAD medium. It is known that *MATa* cells have the capability to produce hyphal protrusions when the α-pheromone gene or *STE12α* is overexpressed in *MATa* cells (6, 42), although these structures are morphologically different from hyphae produced by *MATα* on filament agar. It is not clear, however, why *MATa* cells failed to produce hyphal structures when they were streaked closely to *MATα* cells. The concentration of α-pheromone in such a setting may be inadequate for *MATa* cells to respond.

In *S. cerevisiae*, both pseudohyphal formation and haploid invasive growth require the presence of a functional *STE12* gene (13, 33). In contrast, filamentous growth is only partially defective in *C. albicans* when both copies of *CPH1*, a *STE12* homologue, are disrupted (32). We found that disruption of *STE12α* abolishes hyphal formation on filament agar but only slightly affects hyphal morphology on SLAD medium in the presence of the opposite mating type. Thus, as in *C. albicans* (32), alternative pathways for hyphal formation appear to exist in *C. neoformans*.

Perhaps the most striking observation of this study was that there is strong evidence that *STE12α* is required for virulence. Overexpression of *STE12α* induced the expres-

sion of a number of known virulence genes, and disruption of *STE12α* reduced the expression of these genes. Correspondingly, *ste12α* mutants were less virulent in mice compared with the wild-type, with the difference in a more vigorous immune response to the mutant strain. In fact, the decrease in *ste12α* capsule size in vivo was corroborated by the in vitro GUS data on capsular gene expression in these mutants. Conversely, in strains whose *ste12α* locus was reconstituted, all phenotypes, including virulence (and in vivo capsule size), fertility, phospholipase activity, and haploid fruiting approached wild type. These observations provide an important molecular link between the association of mating type with virulence in *C. neoformans* and support a previous study showing that *MATα* cells are more virulent than *MATa* cells (17).

Since our preliminary study on the effects of the *ste12α* disruptant of serotype D strain was reported (43), Yue et al. have disrupted the *ste12α* gene from the strain H99, a serotype A isolate of *C. neoformans* (44). The *ste12α* mutant of serotype A strain also showed a lack of haploid fruiting and a reduction in mating frequency. The capsule size of *ste12α* mutants was smaller than that of the wild type in vivo and in vitro. Unlike in serotype D isolates, however, phospholipase activity and virulence of *ste12α* mutants were not affected in H99 background. It is most likely that serotype difference was the reason for the discrepancy between our observations and the results of Yue et al. A considerable genetic hiatus between strains of serotype A and D has been observed in sequence differences of rRNA (45) and other genes (46). Our results with serotype D and those of Yue et al. with serotype A suggest possible differences in regulatory pathways. Franzot et al. (46) have even suggested the need for a separate varietal status for *C. neoformans* serotype A isolates.

The phospholipase activity assay, GUS data and virulence studies strongly suggest that the role of *STE12α* in virulence involves the regulation of many of the known virulence-associated genes. Thus, *STE12α* might be a global regulator. However, as deletion of *STE12α* had no visible effect on capsule size and melanin production in vitro or growth rate at 37°C in vitro or in vivo, it is possible that the effect of deleting *STE12α* is too subtle to be detected visually when screened on agar. In this case, the number of genes affected, rather than the expression level of each gene, could function cooperatively to reduce virulence. It is also possible that the regulatory effects of *STE12α* are noticeable only in certain environments, for instance, during stationary phase, reduced $O_2$ tension, increased $CO_2$ concentration, nitrogen starvation, or under certain other stressful conditions. Finally, given that virulence is often multifactorial, *STE12α* may function in the context of one or more of the above scenarios, which would suggest that fungal genes involved in mating have far more diverse roles than merely for reproduction.

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