Developmental Regulation of an Adhesin Gene during Cellular Morphogenesis in the Fungal Pathogen *Candida albicans*\(^{\dagger,+}\)

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*Candida albicans* expresses specific virulence traits that promote disease establishment and progression. These traits include morphological transitions between yeast and hyphal growth forms that are thought to contribute to dissemination and invasion and cell surface adhesins that promote attachment to the host. Here, we describe the regulation of the adhesin gene *ALS3*, which is expressed specifically during hyphal development in *C. albicans*. Using a combination of reporter constructs and regulatory mutants, we show that this regulation is mediated by multiple factors at the transcriptional level. The analysis of *ALS3* promoter deletions revealed that this promoter contains two activation regions: one is essential for activation during hyphal development, while the second increases the amplitude of this activation. Further deletion analyses using the *Renilla reniformis* luciferase reporter delineate the essential activation region between positions −471 and −321 of the promoter. Further 5′ or 3′ deletions block activation. *ALS3* transcription is repressed mainly by *Nrg1* and *Tup1*, but *Rfg1* contributes to this repression. *Efg1*, *Tec1*, and *Bcr1* are essential for the transcriptional activation of *ALS3*, with *Tec1* mediating its effects indirectly through *Bcr1* rather than through the putative *Tec1* sites in the *ALS3* promoter. *ALS3* transcription is not affected by *Cph2*, but *Cph1* contributes to full *ALS3* activation. The data suggest that multiple morphogenetic signaling pathways operate through the promoter of this adhesin gene to mediate its developmental regulation in this major fungal pathogen.

*Candida albicans* is a major opportunistic pathogen of humans (54). This fungus is a frequent cause of superficial oral and vaginal infections, and in immunocompromised patients, *C. albicans* can disseminate via the bloodstream to invade internal organs, thereby causing deep-seated, systemic infections that are often fatal (54).

Various factors are thought to contribute to the virulence of *C. albicans*. These include adhesion to host tissue, the ability to undergo reversible morphogenetic transitions between budding (yeast) and filamentous (hyphae and pseudohyphae) growth forms, the secretion of extracellular hydrolases, and rapid switching between different phenotypic forms (30, 42, 44, 65). The contribution of yeast-hypha morphogenesis to *C. albicans* virulence has been hotly debated (21, 29, 71). However, it is clear that hyphal development is closely associated with tissue invasion (21, 61, 71, 83).

Adherence plays a key role in fungal colonization (27, 68, 70). *C. albicans* expresses an array of adhesin genes including *HWP1*, which encodes a cell surface glycoprotein that acts as a target for mammalian transglutaminases. These enzymes are thought to generate covalent cross-links between Hwp1 on the fungal hyphal surface and proteins on the mammalian cell surface (68, 72). The *ALS* gene family encodes a set of differentially regulated cell surface glycosylphosphatidylinositol-anchored glycoproteins that promote fungal adherence (27, 55). *ALS3* was initially identified as a member of this gene family that is expressed specifically during hyphal development (28).

A second hypha-specific *ALS* gene (*ALS8*) (40) was later identified as an allele of the *ALS3* gene (81). *C. albicans als3/als3* cells are defective in biofilm formation (53, 82). Furthermore, *Als3* is involved in adhesion to endothelial and epithelial cells (55), and *als3/als3* cells display an almost total lack of epithelial destruction in a reconstituted buccal human epithelium model (81). *ALS3* expression has been detected in clinical vaginal fluid specimens and in a vaginal candidiasis model (13). These observations indicate a role for *ALS3* in the pathogenicity of *C. albicans*.

A complex network of signaling pathways regulates yeast-hypha morphogenesis (10). Following exposure to serum, hyphal development is activated by a cyclic AMP-protein kinase A pathway that regulates the activity of the β-helix-loop-helix transcription factor *Efg1* (42, 69). In addition, a mitogen-activated protein kinase pathway, which includes the Ste12-like transcription factor *Cph1*, activates hyphal development under starvation conditions (38, 41). Additional regulatory factors contribute to the activation of hyphal development, but their relationship to these main signaling pathways remains to be understood.
established (11, 16, 39, 57). These include the transcription factors Tec1 and Cph2, the inactivation of which causes defects in hyphal development (37, 62), Tec1 is a TEA (TEF-1, TecIp, and AbaAp)/ATTS (AbaAp, TEF-1, TecIp, and Scalloped) motif transcription factor that is also required for C. albicans virulence (62). It was previously suggested that TEC1 is regulated both by Cph2 and Efg1 (36), but their precise roles in gene regulation during hyphal development are not known.

Hyphal development is negatively regulated by the transcriptional repressors Tup1, Nrg1, and Rfg1 (6, 9, 31, 46). In the absence of hypha-inducing signals, the global repressor Tup1 inhibits the transcription of hypha-specific genes. This repression is dependent upon Nrg1, which binds to Nrg1 response elements (NREs) in the promoters of these genes and targets Tup1 to these promoters (9, 18, 46). Current models suggest that Rfg1 is a second DNA-binding protein that targets Tup1 to the promoters of hypha-specific genes, although Nrg1 appears to make the major contribution to the repression of hyphal development (31, 32).

The prevailing view is that these morphogenetic signaling pathways combine to regulate the transcription of hypha-specific genes. Genome-wide and gene-specific studies have revealed only a small number of hypha-specific genes in C. albicans. These include ALS3, ECE1, HGC1, HWP1, HYR1, RBT1, and RBT4 (2, 5, 8, 28, 48, 67, 83). As described above, ALS3 and HWP1 encode adhesins. The inactivation of RBT1 or RBT4 attenuates C. albicans virulence (9). HGC1 encodes a hypha-specific cyclin required for hyphal development and virulence (83). With the exception of HGC1, all known hypha-specific genes appear to encode secreted or cell wall proteins. These observations reinforce the tight link between the formation of hyphae, the cell surface, and C. albicans virulence.

In this paper, we have examined the organization of the ALS3 promoter and determined the relative contributions of key morphogenetic transcription factors to the regulation of this hypha-specific gene. We find that, relative to other C. albicans genes, the promoter regions of ALS3 and other hypha-specific genes are unusually large. We show that the ALS3 promoter is complex, requiring a 150-bp region for hypha-specific activation. This promoter integrates inputs from multiple activators and repressors. Related observations have been made for a second hypha-specific gene (HWP1) by Kim et al. in the accompanying paper (33).

MATERIALS AND METHODS

Strains and growth conditions. C. albicans strains (Table 1) were grown in YPD at 30°C (63). Hyphal development was induced using 20% bovine calf serum (73).

Strain construction. To generate the C. albicans strains carrying in situ ALS3-GFP promoter fusions, yeast enhanced green fluorescent protein (GFP) was first integrated immediately downstream of the start codon at the ALS3 locus to create SAC500 (Table 1) (14). This was done by PCR amplifying a GFP-URA3 cassette (19) (primers ALS3-F1 and ALS3-F2) (see Table S1 in the supplemental material) and cloning these portions between the ClaI and PstI sites in pCRW3 (66). To test the next sets of ORL promoter fusions, new BstEI, NdeI, SpeI, NotI, and MluI sites were introduced into pCRW3 to make pCRW3N (oligonucleotides KpnSal and SalKpn) (see Table S1 in the supplemental material). A basal ALS3 promoter region (positions −306 to +4) was then PCR amplified and inserted between the PstI and MluI sites in pCRW3N, upstream of the ORL open reading frame (ORF). Various promoter ALS3 fragments were cloned as oligonucleotides or PCR fragments (see Table S1 in the supplemental material) upstream of the basal ALS3 promoter region. The STRE1-, YRE1-, and GRE1-ORL fusions were made by cloning oligonucleotides with each sequence element upstream of a basal ORL reporter containing part of the ADH1 promoter region (51, 74) (see Table S1 in the supplemental material). pCRW3-based plasmids were linearized with HindIII and transformed into C. albicans CABS (Table 1) selecting for the ade2 marker. Single-copy integration at the ade2 locus was confirmed by PCR diagnosis.

To test the roles of Bcr1 and Tec1 in C. albicans, a nonrevertible Ura3− segregant of CJN688 (52) was selected (SAC518) (Table 1). SAC518 was transformed with a GFP-HIS1 cassette (19), as described above, to generate the in situ ALS3-GFP reporter in this ber1 strain (SAC521). Meanwhile, the TEC1 ORF was PCR amplified (primers TEC1-F4 and TEC1-R) (see Table S1 in the supplemental material) and cloned into pPYK1-GFP (45). The resulting plasmid was transformed into the control of the PYK1 promoter. pPYK1-TEC1 and the empty control vector pPYK1 were linearized with StuI and transformed into SAC520 (BCR1) and SAC521 (bcr1) (Table 1). Single-copy integration at RPS1 was confirmed by PCR diagnosis (45).

DNA and RNA analysis. DNA was prepared and analyzed by Southern blotting as described previously (25, 78). RNA was isolated and Northern analysis was performed as described previously (24, 47). The ALS3-specific probe was PCR amplified using primers ALS3-F and ALS3-R, which were described previously by Hoyer et al. (28). GFP and ACT1 sequences were analyzed using probes corresponding to the PCR-amplified ORFs. Primers are specified in Table S1 in the supplemental material.

Reporter assays. GFP fluorescence in whole C. albicans cells was quantified in 96-well, black, clear-bottomed microplates (Matrix Technologies, Wilsom, United Kingdom) using a Tecan Ultra 384 Microplate reader (Tecan Trading AG, Switzerland) running XFluor 4 software. Fluorescence polarization was used to distinguish GFP fluorescence from background autofluorescence (34, 35). The method exploits the high fluorescence anisotropy of GFP compared to other autofluorescing species. The difference between the fluorescence that polarized parallel to the excitation light and that which polarized perpendicular to this excitation light was used as the analytical signal. This measurement is relatively large for GFP and small for autofluorescing molecules. Fluorescence and fluorescence polarization measurements were made at 485-nm excitation and 535-nm emission wavelengths, as described previously (35). Means in “FP [fluorescence polarization] brightness” units and standard deviations from two to eight independent transformants are presented. Observations were reproducible in at least two independent experiments. Untransformed C. albicans cells were used as the background control.

Luciferase assays (relative light units/20 μg protein/20 s) were performed using fresh C. albicans protein extracts with a Lumat LB9507 luminometer (EG&G Berthold) as described previously (46). Means and standard deviations from quadruplicate assays are presented, and similar data were obtained in three experiments using independent transformants.

Microscopy. Cell morphology was monitored using an Olympus BX50 microscope and recorded with an Olympus DP11-P digital video camera. Cell numbers were counted using an Improved Neubauer hemocytometer.

Phase-contrast microscopy and fluorescence microscopy were performed using an Axioscope 2 microscope (Carl Zeiss, United Kingdom) with filter sets X446 (blue emission), X467 (red emission), and X477 (green emission) from Omega Optical Inc. (Brattleboro, VT). Images were generated using a Hamamatsu charge-coupled-device camera and analyzed using Openlab 3.0.9 (Improvision, Coventry, United Kingdom). C. albicans cells were mounted onto polylysine-coated glass slides and covered with Vectashield immunofluorescence mounting medium (Vector Laboratories, Peterborough, United Kingdom) (3).

In silico promoter analysis. Promoter sequences were analyzed for the presence of putative regulatory elements using MatInspector (12, 56) (http://www
| Strain      | Genotype or description | Parent strain | Reference or source |
|------------|------------------------|---------------|---------------------|
| SC5314     | Wild-type clinical isolate |               | 20                  |
| CAI4       | ura3::imm43/ura3::imm43 | SC5314        | This study          |
| CAI8       | ura3::imm43/ura3::imm43 ade2::hisGade2::hisG | CAI4        | 17                  |
| RM1000     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | CAI4        | 50                  |
| BWPI7      | ura3::imm43/ura3::imm43 arg1::hisGarg4::hisG his1::hisGhis1::hisG | RM1000 | 79                  |
| Ca90       | ura3::imm43/ura3::imm43 his1::hisGhis3::hisG | CAI8        | This study          |
| Ca108      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI4        | This study          |
| Ca109      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca110      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI4        | This study          |
| Ca111      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca116      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca117      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca119      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca120      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca122      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca123      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca124      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| Ca125      | ura3::imm43/ura3::imm43 ade2::hisGADE2::ALS3a::RlUC | CAI8        | This study          |
| SAC500     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC501     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC502     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC504     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC505     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC506     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC507     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC508     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC509     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC510     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC512     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC513     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | RM1000 | This study          |
| SAC530     | ura3::imm43/ura3::imm43 ALS3/ALS3-GFP-Ura3 | CAI4        | This study          |
| MMG4       | ura3::imm43/ura3::imm43 arg1::hisGarg2::hisG | CAI4        | 46                  |
| SAC531     | ura3::imm43/ura3::imm43 arg1::hisGarg2::hisG | SAC531      | MMC4                |
| DK158      | ura3::imm43/ura3::imm43 fgl1::hisG | CAI4        | 31                  |
| SAC532     | ura3::imm43/ura3::imm43 fgl1::hisG | DK158       | This study          |
| BCA2-10    | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | CAI4       | 6                   |
| SGC124     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | BCA2-10     | This study          |
| SAC534     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG ALS3/ALS3-GFP-Ura3 | SGC124     | 18                  |
| CHY257     | ura3::imm43/ura3::imm43 a1::hisG | CHY257      | CAI4                |
| JCK18      | ura3::imm43/ura3::imm43 his1::hisG | CAI4        | 41                  |
| SAC536     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | CAI4       | 37                  |
| HLC67      | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | CAI4       | This study          |
| SAC537     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG his2::ARG4p2::his1 | CAI4       | 42                  |
| SAC538     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | HLC67      | This study          |
| SAC540     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | AS18       | This study          |
| SAC539     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | AS18       | This study          |
| CJN702     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | AS18       | This study          |
| SAC519     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | 52                  |
| SAC528     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisGhis1::hisGhis1::hisG | CJN702     | This study          |
| CJN688     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | 52                  |
| SAC518     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisGhis1::hisGhis1::hisG | CJN688     | This study          |
| SAC520     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | This study          |
| SAC521     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | This study          |
| SAC522     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | This study          |
| SAC523     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | This study          |
| SAC524     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | This study          |
| SAC525     | ura3::imm43/ura3::imm43 his1::hisGhis1::hisG | BWP17      | This study          |

*p 5-FOA, 5-fluoroorotic acid.*
**RESULTS**

**ALS3 transcription is activated specifically during hyphal development.** To confirm that the ALS3 gene is expressed specifically during hyphal development in *C. albicans*, we examined ALS3 mRNA levels by Northern blotting following exposure to three distinct types of morphogenetic signals: serum (Fig. 1A), neutral pH, and N-acetylglucosamine (not shown). The ALS3 mRNA was induced in *C. albicans* cells growing at 37°C and strongly induced in cells exposed to serum at 37°C (Fig. 1A). This transcript was undetectable in a *C. albicans* als3/als3 null mutant. ALS3 mRNA levels correlated strongly with the extent of hyphal development in these cultures. The same was true when hyphal development was induced by neutral pH or N-acetylglucosamine (not shown). Our data confirm data from a previous report by Hoyer et al. showing that ALS3 is a hypha-specific gene (28).

To test whether the developmental expression pattern of ALS3 is mediated at a transcriptional or posttranscriptional level, we generated an ALS3-GFP promoter fusion. This promoter fusion was integrated into the *C. albicans* genome in situ at the ALS3 locus (SAC530) (Table 1). The expression of this reporter was monitored by assaying fluorescence levels in *C. albicans* SAC530 cells growing in the presence and absence of serum (Fig. 1B and C). The ALS3-GFP promoter fusion displayed an expression pattern that was similar to that of wild-type ALS3 mRNA, indicating that ALS3 transcription is induced specifically during hyphal development.

**Hypha-specific promoters are unusually long.** Having established that the developmental regulation of ALS3 is mediated at the transcriptional level, we performed an in silico comparison of the ALS3 promoter region and other hypha-specific promoters (*ECE1*, *HGC1*, *HWPI*, *HYR1*, *RBT1*, and *RBT4*). Our aim was to identify common sequence elements that might contribute to the coordinate regulation of these genes during hyphal development. To achieve this, we analyzed the intergenic regions that lie upstream of these genes (Fig. 2). Two main observations were made. First, the 5′-intergenic regions for hypha-specific genes are unusually long compared to *C. albicans* genes in general. The estimated average length of intergenic regions for divergently transcribed *C. albicans* genes is 1,088 bp, that for convergently transcribed genes is 521 bp, and that for tandemly transcribed genes is 770 bp (26). In contrast, the average length of the upstream intergenic regions for these seven hypha-specific genes is 4.5 kbp (based on the latest genome assembly available in the *Candida* Genome Database) (http://www.candidagenome.org/ [accessed October 2006]). The ALS3 intergenic region is 3.0 kbp, and *HCG1* has the longest region at 9.0 kbp. This provided our first clue that morphogenetically regulated promoters in *C. albicans* might be relatively complex. This view is consistent with observations of budding yeast. For example, the developmentally regulated *FLO11* and *HO* genes in *Saccharomyces cerevisiae* both have unusually long and complex promoters (49, 58).

Our second observation was that hypha-specific promoters contain putative binding sites for many known transcription factors in *C. albicans*. These include putative sites for Efg1,
The \textit{ALS3-GFP-URA3} cassette was transformed into wild-type, \textit{ngrl, rfg1, tup1, ssn6,} and \textit{mtla1 mtla2} cells. GFP fluorescence levels were measured in these \textit{C. albicans} strains during growth in the yeast form (Fig. 3B). As expected, the \textit{ALS3-GFP} reporter was repressed in wild-type yeast cells. \textit{Ngrl} acts through two NREs in the \textit{ALS3} promoter at positions $-330$ and $-80$ (46). Hence, the derepression of the \textit{ALS3-GFP} reporter in \textit{ngrl} and \textit{tup1} cells was also expected (Fig. 3B). However, this reporter was only partially derepressed in \textit{rfg1} cells and was not derepressed in \textit{ssn6} or \textit{mtla1 mtla2} cells. These data reinforce the idea that \textit{ALS3} is repressed mainly by \textit{Ngrl} and \textit{Tup1} in an \textit{Ssn6}-independent fashion and that \textit{Rfg1} plays a minor role in the regulation of \textit{ALS3} (18, 32). The data also suggest that although the \textit{ALS3} promoter contains a putative \textit{a1/\alpha 2} site, this repressor is not required for \textit{ALS3} regulation under these conditions.

The \textit{ALS3} promoter also contains putative sites for several transcription factors that are known to contribute to the activation of hyphal development: \textit{Efg1, Cph1, Cph2,} and \textit{Tec1}. Therefore, we examined the contributions of these factors to the activation of \textit{ALS3} expression during hyphal development (Fig. 3). The activity of the in situ \textit{ALS3-GFP} reporter was compared in wild-type, \textit{efg1, cph1, cph2,} and \textit{tec1} cells following serum induction (Fig. 3C). Both \textit{Efg1} and \textit{Tec1} were required for the full activation of the \textit{ALS3-GFP} reporter. In contrast, \textit{Cph2} was not essential for activation, although \textit{Cph2} has been reported to regulate \textit{TEC1} (36). We did observe considerable variation in \textit{ALS3-GFP} expression levels in the \textit{cph2} mutant, and this is reflected in relatively large error bars even though this experiment was performed five times with up to eight independent transformants (Fig. 3C). Decreased \textit{ALS3-GFP} expression was observed in \textit{cph1} cells, suggesting that this mitogen-activated protein kinase pathway does contribute to \textit{ALS3} activation following serum stimulation, although this pathway is not required for hyphal development under these conditions (10, 42). We also examined the impact of \textit{Bcr1} upon \textit{ALS3-GFP}, this is discussed below. Taken together, the data indicate that the transcription factors \textit{Efg1, Tec1, Ngrl,} and \textit{Tup1} play important roles in regulating \textit{ALS3} expression and that \textit{Rfg1} and \textit{Cph1} contribute to \textit{ALS3} regulation.

The \textit{ALS3} promoter contains two main activation regions. A set of mutations was generated at the \textit{ALS3} locus to examine the organization of its promoter. These mutations were generated by inserting a \textit{HIS1} cassette at a range of positions in the 5' intergenic region of the \textit{ALS3-GFP} allele in \textit{C. albicans} strain SAC500 (Table 1). Essentially, this created a set of promoter mutations in situ at the \textit{ALS3} locus, the activities of which were monitored during hyphal development by measuring GFP fluorescence following serum stimulation.

The removal of sequences between positions $-1438$ and $-1049$ (with respect to the first base of the coding region) from the promoter caused a twofold decrease in the activity of the \textit{ALS3-GFP} allele (Fig. 4). The further removal of sequences between positions $-1049$ and $-471$ had no significant effect upon expression. However, the removal of sequences between positions $-471$ and $-306$ blocked \textit{ALS3-GFP} activation completely. We conclude that the full activation of \textit{ALS3} depends upon two promoter regions. One region (A1 [positions $-471$ to $-306$]) is essential for activation, while a second region (A2 [positions $-1438$ to $-1049$]) enhances this activation.
We examined the kinetics of induction of ALS3-GFP transcripts to further investigate the contributions of the A1 and A2 activation regions to the hypha-specific induction of ALS3. Northern analysis was performed on C. albicans SAC501, SAC505, and SAC513 cells following serum stimulation. SAC501 contains both the A1 and A2 activation regions (ALS31499-GFP), and SAC505 lacks A2 but contains A1 (ALS31049-GFP), whereas SAC513 lacks both A1 and A2 (ALS3306-GFP). All three strains developed hyphae at similar rates following serum stimulation, as expected. However, no induction of GFP mRNA was observed for the negative control containing the ALS3306-GFP fusion (Fig. 5). In contrast, GFP mRNA was strongly induced from the positive control containing both activation regions, reaching a maximum at 60 min. Similar kinetics of GFP mRNA induction were observed for the ALS3-GFP construct that contains only the A1 region. However, the GFP mRNA levels reached only about one-third of those in the positive control (Fig. 5), which correlates well with GFP fluorescence levels from these and related constructs (Fig. 4). This reproducible observation was consistent with the idea that the A1 region is essential for transcriptional activation during hyphal development, while the A2 region increases the amplitude of this activation.

The ALS3 promoter is complex. To examine the essential activation region (A1) in more detail, we turned to the sensitive RrLUC reporter (66). First, we tested the robustness of this approach for the dissection of the ALS3 promoter. A set of ALS3-RrLUC promoter fusions containing or lacking the A1 region were integrated into the genome of C. albicans CAI8, and their expression was examined in yeast and hyphal cells. As expected, all of these constructs were inactive in yeast cells (not shown), and only those containing the A1 region (positions −496 to −306) were induced in hyphal cells (Fig. 6A). This indicated that the ALS3-RrLUC fusions accurately reflected the behavior of in situ ALS3-GFP fusions and confirmed the presence of an essential activation region in this part of the promoter.

Additional ALS3-RrLUC constructs were generated to further define the 5’ and 3’ ends of the A1 region. Hypha-specific activation was lost if 5’ sequences between positions −471 and −448 were deleted (not shown). Activation was retained if 3’...
sequences between positions $-321$ and $-307$ were removed, but further 3' deletions to position $-331$ resulted in reduced levels of expression in hyphal cells and the derepression of RrLUC expression in yeast cells. This was consistent with the disruption of activating sequences and the loss of Nrg1-mediated repression through the deletion of the NRE at position $-330$. We concluded that the A1 activation region lies between positions $-471$ and $-321$. This activation region does not correlate well with an in silico analysis of putative regulatory elements in the ALS3 promoter (Fig. 2), reinforcing the view that in isolation, in silico analyses of promoter elements are a poor predictor of regulatory function.

In an attempt to define the A1 region more precisely, we generated a further set of RrLUC constructs containing short overlapping fragments from the A1 region. None of these constructs displayed expression levels equivalent to those of

![Graph showing the analysis of the A1 activation region in the ALS3 promoter.](image-url)
the control (Fig. 6B), indicating that no single enhancer element within the A1 region was sufficient to confer hypha-specific activation. Weak activation (<20% of the control) was observed for some fragments. This might have suggested that multiple copies of a weak element could combine to provide strong activation. However, none of these fragments shared any obvious sequence elements.

Putative binding sites for the transcription factors Msn4/Msn2 (STRE [C₆T]), Cap1 (YRE [TTA[G/C]TAA]), and Gcn4 (GCRE [TGACTC]) do exist in the promoters of hypha-specific genes, and these elements are present in ALS3 promoter fragments that provide weak transcriptional activation. Therefore, we tested whether STRE, YRE, or GCRE elements can activate transcription in response to serum induction (Fig. 6C). The YRE- and GCRE-RtLUC reporters displayed weak activation compared with the ALS3-RtLUC control, suggesting that these elements might contribute to the weak activation seen for the short ALS3 promoter fusions examined in Fig. 6B. However, the YRE element mediates transcriptional activation in C. albicans yeast cells in response to oxidative stress (51), and the GCRE activates transcription in yeast cells in response to amino acid starvation (74). Neither Cap1 nor Gcn4 is required for serum-induced morphogenesis. Hence, these elements cannot account for the hypha specificity of the A1 promoter region. Nevertheless, it is conceivable that YRE and GCRE elements might contribute to the transcriptional activation of hypha-specific genes in the context of the natural promoters.

Taken together, the data suggest that the A1 promoter region is complex. Sequence elements close to the 5’ and 3’ ends of this region are required for the transcriptional activation of ALS3 during hyphal development. These elements appear to function in combination to mediate hypha-specific activation.

**Tec1 acts indirectly through Bcr1 to regulate ALS3 transcription.** Putative Tec1 sites exist in all hypha-specific promoter regions (Fig. 2). Five such sites are present in the ALS3 promoter at positions −1499, −1438, −1049, −885, and −842. Furthermore, Tec1 is required for the morphogenetic activation of ALS3 (Fig. 3C). Therefore, we reasoned that Tec1 might act directly upon the ALS3 promoter via (some of) the putative Tec1 sites. To test this, we generated a set of in situ ALS3 promoter mutants in which the Tec1 sites were sequentially inactivated and compared them to a parallel set of control mutations containing the Tec1 sites (Fig. 4 and Table 1). No significant difference in expression level was observed between each Tec1 site mutation (Fig. 4, gray bars) and its corresponding control (black bars). This indicated that the putative Tec1 sites are not required for the hypha-specific activation of ALS3 and hence that Tec1 might act indirectly upon this gene.

Recently, Nobile and Mitchell (52) identified BCR1 as being a regulator of biofilm formation in C. albicans. During the course of that work, they showed that ALS3 mRNA levels are reduced in bcr1 cells and that BCR1 expression is reduced in a tec1 mutant. This raised the possibility that Tec1 might regulate ALS3 indirectly via Bcr1. We tested this idea by first asking whether BCR1 is required for the transcriptional activation of the ALS3-GFP reporter. ALS3-GFP expression was lost in bcr1 cells, indicating that Bcr1 is essential for the transcriptional activation of ALS3 during hyphal development (Fig. 3C). We then tested whether TEC1 overexpression enhances ALS3 expression and whether this effect is dependent upon BCR1. TEC1 overexpression was engineered by transforming a PYK1-TEC1 fusion into C. albicans SAC520 cells and growing them on glucose-containing medium to activate the PYK1 promoter (4). This led to the significant overexpression of ALS3-GFP (Fig. 7). This overexpression was blocked in a bcr1 mutant background, confirming that Tec1 acts indirectly upon ALS3 transcription via Bcr1.

**DISCUSSION**

Yeast-hypha morphogenesis has been studied intensively in C. albicans because of its likely contribution to the pathogenicity of this fungus (21, 22, 29, 61, 71, 83). A complex network of signaling pathways has been shown to control hyphal development, but the mechanistic relationships between these pathways remain obscure (10). These signaling pathways are thought to converge on the promoters of those genes that respond specifically during hyphal development (7, 10). ALS3 is one of a small set of hypha-specific genes in C. albicans that includes ALS3, ECE1, HGC1, HWP1, HYR1, RBT1, and RBT4 (2, 5, 8, 28, 40, 48, 67, 83). In this study, we have confirmed that the hypha-specific activation of ALS3 is mediated at the transcriptional level (Fig. 1). Clearly, a complete understanding of morphogenetic signaling depends upon the dissection of hypha-specific promoters and the mechanisms by which these pathways regulate these promoters.

We have shown that ALS3 is regulated by a complex array of transcription factors: Efg1, Cph1, Tec1, Bcr1, Nrg1, Rfg1, and Tup1 (Fig. 3). When C. albicans cells grow in the yeast form, ALS3 transcription is repressed mainly by Nrg1, which binds to NREs located at positions −330 and −80 in the promoter (46). Rfg1 also contributes to ALS3 repression (Fig. 3) (32), but the promoter element(s) through which Rfg1 operates in C. albicans has not been experimentally defined. Both Nrg1 and Rfg1 are thought to act by interacting with the global repressor Tup1, which mediates transcription through direct interactions between each Tec1 site mutation (Fig. 4, gray bars) and its corresponding control (black bars). This indicated that the putative Tec1 sites are not required for the hypha-specific activation of ALS3 and hence that Tec1 might act indirectly upon this gene.

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with the transcription complex, by positioning nucleosomes on the promoter, or by a combination of both mechanisms (23, 80). In S. cerevisiae, interactions between Tup1 and its cognate DNA binding proteins often depend on Ssn6 (64). However, this does not appear to be the case for Nrg1 in the context of hypha-specific genes. It has been suggested that the repression of hypha-specific genes by Nrg1 and Tup1 does not depend upon Ssn6 (18), and we have confirmed this for ALS3 in this study (Fig. 3).

Cph1 and the A2 region of the promoter are required only for full ALS3 activation. This might suggest that Cph1 enhances ALS3 transcription via the A2 region. However, there are no obvious occurrences of the putative Cph1 consensus site in the ALS3 promoter, and therefore, Cph1 might act indirectly to regulate ALS3 transcription (Fig. 8).

In contrast, Efg1 is essential for the transcriptional activation of ALS3 during hyphal development (Fig. 3C). Efg1 has been shown to bind an E box in vitro (40), and hypha-specific promoters do contain this type of sequence element (Fig. 2). However, the degenerate E-box consensus is likely to occur frequently by chance (1/256), and to date, there are no reports confirming that Efg1 regulates transcription via the E box in C. albicans.

Although Tec1 is essential for the activation of ALS3 (Fig. 4) and putative Tec1 sites exist in the ALS3 promoter (Fig. 2), these sites do not contribute significantly to ALS3 activation (Fig. 4). Instead, Tec1 regulates ALS3 transcription indirectly through Bcr1 (Fig. 7), which is also essential for ALS3 activation (Fig. 3). These observations are entirely consistent with recent data from Nobile et al. They showed that Tec1 and Bcr1 are required for the formation of biofilms in C. albicans and that Bcr1 acts downstream of Tec1 to regulate the expression of adhesin genes required for biofilm formation, such as ALS3 and HWP1 (52, 53).

The transcriptional activation of ALS3 is dependent upon the A1 promoter region (Fig. 4) as well as upon Efg1, Tec1, and Bcr1 (Fig. 3). The A1 promoter region is complex: no single sequence element within this 150-bp region was capable of driving hypha-specific expression, and the trimming of sequences at either the 5’ or 3’ end of this A1 region blocked hypha-specific activation (Fig. 6). This is consistent with the idea that several different regulatory factors converge upon the A1 region to cooperate in ALS3 activation. Hence, Tec1-Bcr1 and Efg1 might regulate ALS3 cooperatively via the A1 promoter region (Fig. 8). An NRE lies at the 3’ border of the A1 region at position −330. It has been reported that Nrg1 might act as a transcriptional activator under some circumstances (47, 59). Hence, it is conceivable that Nrg1 might also contribute to the hyphal activation of ALS3.

In parallel studies, Kim and coworkers (33) made similar observations about the regulation of a second hypha-specific gene, HWP1. The HWP1 promoter also contains two activation regions. One region, which binds an array of chromatin remodeling proteins, is essential for HWP1 activation, whereas the second distal region increases the amplitude of this activation (33). Hence, this class of developmentally regulated genes appears to be controlled by complex interactions between several critical transcription factors at the level of their promoters. It has long been recognized that C. albicans responds to an extremely disparate range of environmental conditions by forming hyphae (54). The unusual length of promoters of hypha-specific genes and the complexity and diversity of factors regulating their transcription not only are compatible with the diversity of conditions known to favor hypha formation but also suggest that morphogenetic changes in C. albicans may be affected by events in several regulatory pathways whose stimulation may not always be specifically or directly related to cell shape.

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