The DNA Binding Protein Rfg1 Is a Repressor of Filamentation in *Candida albicans*

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ABSTRACT

We have identified a repressor of hyphal growth in the pathogenic yeast *Candida albicans*. The gene was originally cloned in an attempt to characterize the homologue of the *Saccharomyces cerevisiae* Rox1, a repressor of hypoxic genes. Rox1 is an HMG-domain, DNA binding protein with a repression domain that recruits the Tup1/Ssn6 general repression complex to achieve repression. The *C. albicans* clone also encoded an HMG protein that was capable of repression of a hypoxic gene in a *S. cerevisiae rox1* deletion strain. Gel retardation experiments using the purified HMG domain of this protein demonstrated that it was capable of binding specifically to a *S. cerevisiae* hypoxic operator DNA sequence. These data seemed to indicate that this gene encoded a hypoxic repressor. However, surprisingly, when a homozygous deletion was generated in *C. albicans*, the cells became constitutive for hyphal growth. This phenotype was rescued by the reintroduction of the wild-type gene on a plasmid, proving that the hyphal growth phenotype was due to the deletion and not a secondary mutation. Furthermore, oxygen repression of the hypoxic *HEM13* gene was not affected by the deletion nor was this putative *ROX1* gene regulated positively by oxygen as is the case for the *S. cerevisiae* gene. All these data indicate that this gene, now designated *RFG1* for *R*epressor of *F*ilamentous *G*rowth, is a repressor of genes required for hyphal growth and not a hypoxic repressor.

MANY yeast species have the capacity to grow under strongly hypoxic conditions. Cells adapt to hypoxia by inducing genes that encode functions that enable them to use limiting oxygen more efficiently. We have studied the regulation of these hypoxic genes extensively in one yeast, the model organism *Saccharomyces cerevisiae* (for review see Zitomer and Lowry 1992; Zitomer *et al.* 1997; Kastaniotis and Zitomer 2000). Oxygen levels are sensed in the cell by the levels of heme; heme biosynthesis requires oxygen as substrate at two steps. Heme serves as a cofactor for the transcriptional activator Hap1, which activates the expression of aerobically induced genes. One of these genes is *ROX1*, which encodes a repressor of the hypoxic genes. Thus, under aerobic conditions, heme accumulates, the transcription of *ROX1* is activated, and the hypoxic genes are repressed. Under hypoxic conditions, heme levels are reduced, *ROX1* is not transcribed, and the hypoxic genes are derepressed.

Rox1 consists of 368 amino acids (Balasubramanian *et al.* 1993). The first fourth of the protein comprises an HMG domain, a DNA binding and bending motif (Balasubramanian *et al.* 1993; Deckert *et al.* 1995b, 1999). Through this domain, Rox1 binds to a specific DNA sequence located upstream of the hypoxic genes. The last two-thirds of the protein comprises the repression domain. This domain recruits the Tup1/Ssn6 general repression complex to the hypoxic genes to achieve repression (Deckert *et al.* 1995b). The Tup1/Ssn6 complex is involved in the repression of a diverse set of regulons in *S. cerevisiae* (Mukai *et al.* 1991; Williams *et al.* 1991; Zhang *et al.* 1991; Keleher *et al.* 1992; Elledge *et al.* 1993; Teunissen *et al.* 1995; Friesen *et al.* 1997; Marquez *et al.* 1998; Mizuno *et al.* 1998). The complex has no intrinsic DNA binding activity, but is recruited to target genes by regulon-specific DNA binding repressor proteins (Keleher *et al.* 1992; Tzamarias and Struhl 1994, 1995; Treitel and Carlson 1995). It is the regulation of the synthesis or activity of each of these regulon-specific proteins that achieves the desired pattern of gene expression for each regulon.

In our genetic analysis of the Rox1 protein, we have isolated a large number of mutations in the HMG domain and characterized their effects on DNA binding and bending *in vitro* and repression *in vivo* (Deckert *et al.* 1999). However, the analysis of the repression domain has proved frustrating. This domain is functionally redundant; either half of this domain can support repression, and deletion analysis revealed no specific essential elements (Deckert *et al.* 1995b). The amino acid sequence of this domain has no clear repeated elements and provides no clues to its functional redundancy. Also, it has been difficult to establish an assay for the association of Rox1 with the Tup1/Ssn6 complex *in vitro,* which would help define essential elements of

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the Rox1 protein. Therefore, we have turned to a comparative sequence analysis approach, anticipating that the residues required for the interaction with Tup1/Ssn6 would be conserved in the Rox1 homologues of other yeast species.

We report here our attempt to clone the *ROX1* homologue from *Candida albicans*. We began with this yeast for several reasons. First, it can grow under hypoxic conditions and, therefore, is likely to have a hypoxic regulon. Second, this yeast is phylogenetically close enough to *S. cerevisiae* to expect conservation of general regulatory mechanisms. Indeed, this conservation has been extensively exploited in the study of filamentous growth in *C. albicans* (for reviews see Corner and Magee 1997; Kobayashi and Cutler 1998; Mitchell 1998; Brown *et al.* 2000). However, the two yeasts are sufficiently distantly related to expect divergence of amino acid residues that are not under strong selective pressure. Third, there is an ongoing sequence project for the *C. albicans* genome that proved helpful in identifying a putative *ROX1* homologue and would be helpful in identifying hypoxic target genes. Finally, *C. albicans* is a human pathogen, a major killer of immune-compromised patients, and we suspected there might be a link between pathogenicity and hypoxia. Pathogenicity is strongly linked to filamentous growth, which may be important for tissue invasion. Hypoxia has been reported to be one of the many signals that triggers the transition to filamentous growth (Odds 1985), albeit a weak one. Also, as hyphae invade tissue, hypoxia may be one of the environmental changes incurred. Thus, we felt that an analysis of hypoxia in *C. albicans* may have some relevance to pathogenicity.

We identified a *C. albicans* gene with extensive similarity to the *S. cerevisiae ROX1* gene. We demonstrated that it bound specifically to the hypoxic target DNA sequence and that it served as a hypoxic repressor in *S. cerevisiae*. However, to our surprise, we found that deletion of the gene caused constitutive hyphal growth and that it did not regulate the *HEM13* hypoxic gene of *C. albicans*. Therefore, this gene appears to encode a repressor of hyphal growth rather than a hypoxic repressor.

MATERIALS AND METHODS

Yeast strains and cell growth: The *C. albicans* strain RM1000 was used in this study (Negredo *et al.* 1997). The *S. cerevisiae* strain MZ22-4Δ*r1::gK* contains a deletion of the *ROX1* gene and an integrated *ANB1-lacZ* fusion (Deckert *et al.* 1999). MZ22-4Δ*r1*Δ*m3* is a derivative of MZ22-4Δ*r1::gK* containing a deletion of the *MOT3* gene (Kastaniotis *et al.* 2000). MZ22-4PC is a derivative of MZ22-4Δ*r1::gK* containing the *tup1::TRP1* allele.

Cells were maintained and grown without selection on rich YPD medium (Kaiser *et al.* 1994). SC synthetic medium lacking specific nutrients was used for selective growth (Kaiser *et al.*, 1994). For aerobic growth for RNA preparations, cells were grown on YPD with vigorous shaking at 30°. For short-term anaerobic growth for RNA preparations, ultrapure nitrogen (99.9%) was bubbled through cultures for 2 hr, vigorously at first, and then more slowly to maintain anaerobic conditions. For β-galactosidase assays, cells were grown in SC selective media at 30° with vigorous shaking. For long-term anaerobic growth for β-galactosidase assays, cells were grown in SC selective media supplemented with 20 μg/ml ergosterol and 0.2% Tween 80 overnight in flasks packed into anaerobic chambers with a GasPak kit (BBL). To induce filamentous growth in *C. albicans*, cells were grown in 10% fetal calf serum overnight with vigorous shaking at 30°. Cells were stained with calcofluor as described (Adams and Pringle 1991).

Plasmids: All plasmids were maintained in *Escherichia coli* strain HB101 as described (Ausubel *et al.* 2000). Plasmid constructions and enzymatic manipulations were carried out using standard procedures (Ausubel *et al.* 2000) and conditions recommended by the enzyme vendors. The sequences of synthetic oligonucleotides used for PCR and sequence analyses are available upon request.

The *C. albicans* library in pEMBLy23 was provided by P. T. Magee (University of Minnesota) and a second library was provided by G. R. Fink (MIT). The *C. albicans* transforming plasmid pABSK2 carrying the *C. albicans URA3* selectable marker and the *ARS2* replication origin was provided by H. Chibana (University of Minnesota). Plasmids pGEM-*URA3* and pGEM-*HIS1* containing the *C. albicans URA3* and *HIS1* genes, respectively, were obtained from Dr. A. P. Mitchell (Columbia) (Wilson *et al.* 1999).

The plasmids below were constructed for this study. The sequences are numbered with the A of the ATG translational initiation codon as +1, and sequences 3′ are numbered consecutively in positive integers and sequenced 5′ in negative integers.

pBS-*ROX1*, the initial clone of the *C. albicans ROX1* N-terminal coding region, was obtained by PCR using primers homologous to the ends of the sequence present in the *C. albicans* database (http://www.alces.med.umn.edu) at that time. It contains residues from +3 to +321 flanked by the restriction sites *Kpn*I and *Hin*dIII.

p*ROX1*-HMG was identified from the library of Dr. G. R. Fink by colony hybridization using the insert from pBS-*ROX1*. It contained the sequences from about −1000 to +600.

p*ROX1*-C was obtained by amplification of cDNA prepared from *C. albicans* poly(A) RNA using an oligo(dT) primer and a *ROX1* specific primer from +495 to +519. The PCR product was digested with *Hin*dIII and *Xba*I and ligated into the complementary sites of pBSK (Stratagene, La Jolla, CA).

YCp(22)Ca*ROX1* contained a genomic copy of the *ROX1* gene. The PCR fragment containing residues −574 to +1314 flanked by *Hin*dIII and *Bst*EII restriction sites was ligated to the homologous sites of YCp(22)*ROX1*Hx (Deckert *et al.* 1995a), replacing the *S. cerevisiae ROX1* upstream and coding sequences but retaining the 3′ sequences.

YCp(33)*CaROX1* is identical to YCp(22)*CaROX1* except that the *CaROX1* gene is contained in the vector YCplac33 (Gietz and Sugino 1988).

YCp(22)*ROX1*EKP contained the *S. cerevisiae ROX1* on a 2.8-kb fragment with an *Eco*RI site at the 5′ end, a *Kpn*I site at codons 2 and 3, and a *Pst*I site at the 3′ end. It was constructed by two separate PCR reactions (*Eco*RI to *Kpn*I and *Kpn*I to *Pst*I) followed by a three-way ligation into the *Eco*RI-*Pst*I sites of YCplac22 (Gietz and Sugino 1988).

YCp(22)Ca*R1*-HMG1 contained a replacement of the sequences encoding the *S. cerevisiae* Rox1 HMG-domain (codons 2 to 123) with those of the *C. albicans* Rox1 HMG-domain (codons 38 to 119). It was constructed by PCR

amplification of *C. albicans ROX1* sequences from p*ROX1*-HMG using primers that placed a *Kpn*I site at the 5′ end and an *Xho*I site at the 3′ end of the coding sequences. The fragment was ligated with YCp(22)*ROX1*EKP digested with *Pst*I and *Kpn*I plus the *Xho*I-*Pst*I fragment from YCp(22)*R1-100/123* (Deckert *et al.* 1995b).

YCp(22)Ca*R1*-HMG2 was similar to YCp(22)Ca*R1*-HMG1 but contained the *C. albicans ROX1* sequences from 38 to 199. It was constructed in the same fashion as above.

YCp(33)Ca*R1*-C contained a replacement of the sequences encoding the *S. cerevisiae* Rox1 repression domain (codons 100 to 360) with those of the *C. albicans* Rox1 repression domain (codon 165 to 30 bp beyond the termination codon). It was constructed by PCR amplification from p*ROX1*-C using primers that placed an XhoI site at the 5′ end and a *Bst*EII site at the 3′ end. This fragment was ligated into plasmid YCp(33)*R1*Δ*100/245* (Deckert *et al.* 1995b) digested with *Xho*I and *Bst*EII.

pMAL-*CaROX1(HMG)* contained a fusion of the maltose binding protein gene *malE* to the HMG-domain encoding sequences of the *C. albicans ROX1* gene from codons 38 to 174. It was constructed by PCR amplification of the *ROX1* HMG domain from pBS-*ROX1* using primers that placed *Kpn*I and *Pst*I restriction sites at the 5′ and 3′ ends of the PCR fragment, respectively. The fragment was ligated into pMAL-*ROX1*(HMG) (Deckert *et al.* 1999) digested with the same enzymes.

YCAp*ROX1* contains the *C. albicans ROX1* gene from −574 to +1314 inserted into the *URA3*-containing *C. albicans* transforming plasmid pABSK2. It was constructed by inserting the *Hin*dIII-*Kpn*I fragment from YCp(22)Ca*ROX1* into the homologous sites of pABSK2.

Sequence analysis: The sequence of both strands of the *C. albicans ROX1* gene was determined using the Sequenase kit (United States Biochemical, Cleveland) and synthetic primers.

Construction of deletion strains: The deletions of the *ROX1* gene in *C. albicans* were generated by transforming cells with PCR fragments. The *URA3* deletion allele fragment was generated using one synthetic primer that contained the *ROX1* sequences from +90 to +141 followed by *URA3* sequences from −404 to −382 and a second primer containing the *ROX1* sequences from +1314 to +1366 followed by *URA3* sequences from +920 to +942. These primers were used to PCR amplify the *URA3* gene from pGEM-*URA3*, resulting in a fragment containing the *URA3* gene flanked by the *ROX1* sequences of the primers. *C. albicans* strain RM1000 was transformed with this fragment as described (Wilson *et al.* 1999).

The *HIS1* deletion allele was generated in a similar fashion except that the primers contained *HIS1* sequences (−337 to −317 for one primer and 900 to 916 for the other) at the 3′ end following the *ROX1* sequences. The *HIS1* sequences were amplified from pGEM-*HIS1*.

The correct constructs were confirmed by PCR analyses of genomic DNA. The strategy involved the use of four sets of primers. The first set hybridized to sequences internal to the deleted *ROX1* sequences such that only the wild-type allele would generate a PCR product. For the second set of primers, one hybridized outside the deleted *ROX1* sequences and the other hybridized to one end of the *URA3* sequences such that a PCR product would be generated only from the *rox1::URA3* allele. The third set was similar to the second, except a *HIS1* primer was used instead of the *URA3* primer; this set was specific for the *rox1::HIS1* allele. Finally, a set of primers that amplified the actin gene was used as a control for the quality of the DNA preparations. The data generated with these primers have been reviewed by the Communicating Editor.

Protein purification and DNA binding analysis: The HMG domain of the *C. albicans* Rox1 protein was expressed from *pMAL-CaROX1(HMG)* in the *E. coli* strain PR745 (New England Biolabs, Beverly, MA) as a fusion to the maltose binding protein (MBP). The protein purification procedure was identical to that described for the *S. cerevisiae* MBP-Rox1(HMG) fusion (Balasubramanian *et al.* 1993). The gel retardation assay was carried out as described previously (Balasubramanian *et al.* 1993).

β-Galactosidase assays: The assays for β-galactosidase expression in yeast were carried out as described (Kaiser *et al.* 1994). The activity is expressed as Miller units.

Preparation of RNA, reverse transcription, and RT-PCR: RNA was prepared from yeast cells as described (Lowry and Zitomer 1984). Where indicated, poly(A) RNA was prepared using oligo(dT) sepharose affinity chromatography (Ausubel *et al.* 2000). cDNA was generated using reverse transcriptase as described (Ausubel *et al.* 2000), and the RNA was removed by treatment with DNase free RNase (Roche Biochemicals). PCR was carried out using the primers indicated for varying numbers of cycles to ensure that product formation was within the linear range. For each RNA preparation, amplification without reverse transcription was carried out with each set of primers to ensure that there was no genomic DNA contamination.

RESULTS

Cloning and sequence of the *C. albicans ROX1* gene: The cloning of the *C. albicans ROX1* gene was based upon the appearance of a 320-bp DNA sequence with similarity to the HMG-domain coding region of the *S. cerevisiae ROX1* gene and annotated as a *ROX1* homologue in the *C. albicans* database maintained at the University of Minnesota. PCR primers were generated to the ends of this sequence, and it was amplified, subcloned, and used as a probe in colony hybridization to two different libraries of *C. albicans* genomic DNA. Positive clones were sequenced and contained only the HMG domain and upstream sequences; the C-terminal coding sequence could not be found in either library. To obtain this region, RNA was prepared from *C. albicans* cells, and cDNA was generated to total poly(A) containing RNA. The *ROX1* C-terminal coding region and the 3′-untranslated region was amplified from this cDNA pool using oligo(dT) and a *ROX1*-HMG specific primer. The PCR products were cloned and sequenced, and those containing the continuation of the *ROX1* coding sequence were identified from the overlap with the previously sequenced regions. Finally, the genomic sequences from −547 through the 3′-untranslated region were cloned by PCR. For the purposes of this study, the *C. albicans* gene is referred to as *ROX1* and the *S. cerevisiae* gene is referred to as *ScROX1*.

The complete DNA sequence from −547 to +1366, where the A of the first ATG initiation codon in the open reading frame is +1, has been deposited in the EMBL database. The protein coding sequence for the HMG domain from codon 1 to 189 is presented in Figure 1. The core amino acid sequence of the HMG domain from residues 30 to 119 is aligned with that of

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MSIGGGIDGNFSHNNNNNNNNNNNNNNNKCICKSKVNKIPRPRNAFILFRQKYHQMVLDE  60
                              ||   |||||:|||||||:||:::::||
                            MNPKSSTPKIPRPKNAFILFRQHYHRILIDE

----GTVIRTNPEVSRELGRRWRGLSPQEKEHWNNLAEEEKNNRAKKYPGYRYTPRRNGR  120
    |  |  | ::|: :| :|:|| | :| ||:|||| || :: :||| |:| | |: :
WTAQGVEIPHNSNISKIIGTKWKGLQPLDKAHWENLAEKEKLEHERKYPEYKYKPVRKSK

NKNCPVCKNKPLPNNKSNSISGMSGSGGGGGSISGASSLSGGLTSRDNSTTNANAIDYQQ  180
:|:
KKQ

QQLQQQQQQQQQ
```

FIGURE 1.—Comparison of the *C. albicans* and *S. cerevisiae* Rox1-HMG domains. The *C. albicans* Rox1 protein sequence from residues 1 to 189 is shown in boldface type; the *S. cerevisiae* Rox1 sequence from residues 1 to 94 is indicated in lightface type. Identical residues are designated by a vertical line and conserved residues by a colon. The dashed lines represent four residues present in the *S. cerevisiae* sequence that are missing from the *C. albicans* protein.

the ScRox1 HMG domain from residues 1 to 94. The two sequences are 50% identical and 74% conserved over this region. The most conserved region lies between the *C. albicans* residues 38 to 60, where 17 of the 23 residues are identical and the remainder are highly conserved substitutions. This region represents the first helix of the HMG domain that makes the majority of the specific contacts with DNA (WERNER *et al.* 1995; DECKERT *et al.* 1999). This region ends in a loop before the second helix which varies in length among the different HMG domain proteins (GROSSCHEDL *et al.* 1994), and the ScRox1 protein has four additional residues in this region compared to the *C. albicans* Rox1. In the ScRox1, the HMG domain begins shortly after the initiation methionine and ends shortly before a run of glutamine residues, but the *C. albicans* protein has extensive additions at both ends. It is likely that these sequences are translated because (1) they appear in the cDNA and (2) the extension at the carboxyl end of this domain is required for Rox1 function in *S. cerevisiae* cells (see below).

The C-terminal region, which we presume contains the repression domain based upon complementation studies below, has little homology to the ScRox1 repression domain. This observation was not surprising because our previous genetic studies with the *S. cerevisiae* repression domain suggested that it did not require a highly conserved sequence. Rather, the region is redundant in function but not in sequence. Nonetheless, there are small pockets of sequence similarity between this region in the two Rox1 sequences, although their significance is unclear at this time.

The *C. albicans ROX1* complements a *rox1* deletion in *S. cerevisiae*: To determine whether the cloned *C. albicans* gene functions as a DNA binding repressor protein, we determined its ability to complement a *rox1* deletion allele in *S. cerevisiae*. Complementation was measured by the ability to repress the *ANB1-lacZ* fusion, a reporter hypoxic gene. We generated a number of constructs that tested the ability of the HMG domain, the repression domain, and the full-length protein to function in the heterologous species. As can be seen in Table 1, a construct consisting of the HMG domain from residues 38 to 199 fused to the ScRox1 repression domain and, driven by the *ScROX1* promoter, was capable of repression of the reporter (line 3). However, surprisingly, a shorter region of the HMG domain, lacking residues 120 to 199 that are not present in the ScRox1 and not part of the basic HMG domain, was not capable of complementation (line 2), indicating that this region plays some important but unknown role in Rox1 DNA binding or protein stability that is not required in the ScRox1.

Also evident from Table 1, the C-terminal sequences of the *C. albicans* Rox1 contain a repression domain. The fusion protein containing this region joined to the ScRox1 HMG domain was able to repress the reporter gene (line 4). Therefore, despite the extensive sequence divergence, this region is still recognized as a functional repression domain in *S. cerevisiae*.

Finally, the intact *ROX1* gene, including the regulatory sequences, complemented the *rox1* deletion, demonstrating that the intact gene encodes an authentic DNA binding repressor protein (Table 1). Furthermore, the *ROX1* gene did not repress anaerobic *ANB1-lacZ* expression. *ScROX1* expression is regulated by oxygen, and constitutive expression results in both aerobic and anaerobic repression of the hypoxic genes (KENG 1992). Although the anaerobic expression of the reporter gene suggested that the *C. albicans* promoter sequences are regulated by oxygen in *S. cerevisiae*, experiments detailed below demonstrate that such was not the case. The level of repression by all of the *ROX1* constructs was threefold weaker than that observed for the native *ScROX1* gene, and this effect was due to a second hypoxic regulator, Mot3 (see below).

The HMG domain binds to the Rox1 consensus sequence: The cross-species complementation experiments indicated that the Rox1 HMG domain bound to the ScRox1 target site. To confirm this conclusion directly, the ability of the Rox1 HMG domain to bind the ScRox1 consensus sequence *in vitro* was determined. The *ROX1* HMG-domain coding sequence from codons 38 to 174 was fused to the *E. coli malE* gene, encoding the MBP, and the fusion protein was expressed in and purified from bacterial cells. Gel retardation studies using this fusion protein indicated that the protein bound to the ScRox1 binding site (Figure 2, lanes 2, 3, and

TABLE 1

Repression of *S. cerevisiae* Hypoxic genes by ScRox1-CaRox1 fusions

Plasmid[a]	Fusion gene[b]			β-Galactosidase activity[c]	
	Regulatory region	HMG domain	C-terminal domain	Aerobic	Anaerobic
YCp(22) *ROX1*H	Sc	Sc	Sc	6.5	109
YCp(22) *Ca-R1*	Sc	*Sc* 1, *Ca* 38-119	Sc	43	—
YCp(22) *Ca-R1*HMG2	Sc	*Sc* 1, *Ca* 38-199	Sc	18	—
YCp(22) *Ca-R1*-C	Sc	Sc	*Ca* 165-C term.	18	—
YCp(22) *CaROX1*	Ca	Ca	Ca	26	126
YCplac22 (Vector)	—	—	—	143	126

[a] Plasmids were transformed into MZ22-4Δ*r1::gK*.

[b] *S. cerevisiae* regions of the fusion are indicated by the abbreviation *Sc*; *C. albicans* regions are indicated by the abbreviation *Ca*. Where indicated, the residue numbers represent codons. The construction of the fusions are presented in the MATERIALS AND METHODS.

[c] β-Galactosidase was expressed from an integrated copy of the *ANB1-lacZ* fusion in MZ22-4Δ*r1::gK*. Dashed lines indicate that the assays were not carried out under anaerobic conditions.

4). Binding was specific for sequence containing the ScRox1 binding site as determined by competition with either a sequence containing two Rox1 sites of the *ANB1* OpA (lane 5 and 6), which decreased the amount of labeled complex visible, *or* a similar sequence with a deletion of the ScRox1 binding sites (lane 7), which did not reduce the amount of labeled complex.

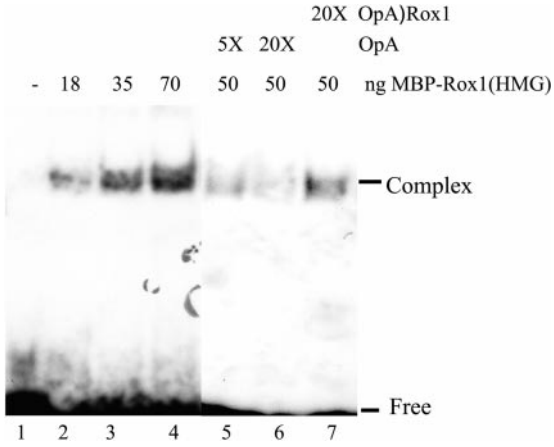

FIGURE 2.—The *C. albicans* Rox1 HMG domain binds to the ScRox1 binding site. A gel retardation assay was performed with purified MBP-Rox1(HMG) protein. The labeled DNA fragment used was generated by annealing two complementary fragments, which left 5′ single-stranded ends that were filled in with [^{32}P]dATP(AUSUBEL et al. 2000). When filled in, the sequence of the top strand was 5′ GGGTTTTCAG**CCCATTGTTCTC**GAGCAAACC, where the sequence in boldface represents the Rox1 binding site (DECKERT et al. 1998). Lane 1 contained no protein, and the nanograms of protein added to each sample are indicated above the lanes. Competitor DNAs were added to lanes 5 – 7 in the fold-excess of labeled DNA indicated. The top strand of the double-stranded specific DNA competitor sequence, OpA, was 5′ TTTT**TCCATTGTTCGT**TGCTTGCCTGTTTTTTTGC**CCTATTGTTCTC**AAAA. This sequence contained two Rox1 binding sites (boldface). The nonspecific competitor, OpAΔRox1, contained the same sequence except that the underlined bases, composing the core of the Rox1 site, were deleted.

Deletion of *ROX1* results in constitutive filamentous growth: To determine whether the *ROX1* gene regulates the hypoxic genes in *C. albicans*, it was necessary to generate a deletion strain. Since *C. albicans* is diploid with no known meiotic division, the deletion had to be constructed in each homologue. This was achieved by successive gene replacements using a *ura3/ura3 his1/his1* strain. One copy of the gene was precisely deleted and replaced with the *URA3* gene to generate the heterozygote, and then the second copy was precisely deleted and replaced with the *HIS1* gene. Two independent homozygous deletion clones were obtained and, surprisingly, the colony morphology of both was quite irregular. Microscopic inspection revealed that the cells grew as hyphae in rich medium as opposed to the budding growth of the wild-type and heterozygote strains (Figure 4). Hyphal growth is normally triggered only under specific conditions, most strongly in serum (ODDS 1985; Figure 3). These results suggest that *ROX1* is a repressor of hyphal growth.

Given that we isolated only two homozygous deletion strains and that it had an unexpected phenotype, we attempted to reisolate the double deletion, but this time first generating the *HIS1* replacement. By extraordinary luck, often better than careful experimental design, one His$^+$ transformant had the irregular colony morphology and, when analyzed by PCR, was found to be homozygous for the *HIS1* replacement. Again, these cells grew as hyphae (Figure 3). Since these cells were still *ura3* auxotrophs, we transformed them with a *URA3* vector carrying the wild-type *ROX1* gene. These transformants reverted back to a budding growth pattern (Figure 3), although there were many cells that appeared to be initiating hyphal growth, probably due to the unstable nature of the plasmid. (Since cell growth was main-

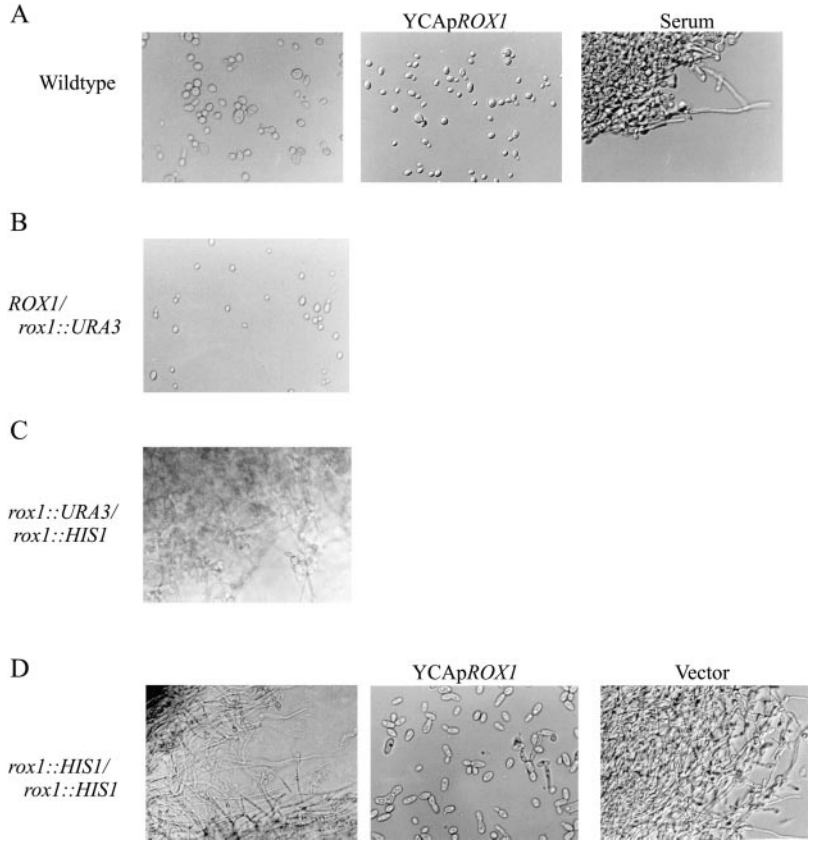

FIGURE 3.—A *ROX1* deletion causes hyphal growth. Cells were grown aerobically at 30°. The magnification was ×400. (A) RM1000 (wild-type) cells were untransformed grown in SC medium (left), transformed with YCAp*ROX1* and grown in SC-uracil (middle), or untransformed grown in 10% fetal calf serum (right). (B) RM1000 *ROX1/rox1::URA3* heterozygotes were grown in SC medium. (C) RM1000 *rox1::URA3/rox1::HIS1* mutants were grown in SC medium. (D) RM1000 *rox1::HIS1/rox1::HIS1* were untransformed and grown in SC medium (left), transformed with YCAp*ROX1* and grown in SC-uracil, and transformed with pABSK2 (Vector) and grown in SC-uracil.

tained under selective conditions, growth of those cells that lost the plasmid could not continue and give rise to hyphae.) The double deletant transformed with the vector alone remained filamentous (Figure 3). Given that independent isolates of the homozygous deletion yielded the same hyphal growth phenotype and that this phenotype could be suppressed by transforming cells with a wild-type copy of the gene, we conclude that *ROX1* encodes a repressor of hyphal growth.

To determine whether the homozygous *rox1* deletant formed pseudo- or true hyphae, we stained cells with calcofluor, a chitin-specific fluorescent dye that reveals the septum between true hyphal cells. As can be seen in Figure 4, the deletant culture grown on rich medium contained a mixture of cells; single budded cells, pseudohyphae (Figure 4A), and true hyphae (Figure 4, A and B) were all visible. The (morphological index) value for the filamentous cells shown in Figure 4B averages 3.4–3.9 with an average of 3.7, close to the value expected for true hyphae (MERSON-DAVIES and ODDS 1989). It was difficult to obtain an accurate assessment of the percentages of cell types because the hyphal mat was too thick and too difficult to break apart to allow an accurate, unbiased count. The cells shown in Figure 4 represent a combination of free cells and those that broke off from the mat after vigorous mixing. Nonetheless, it was obvious that most cells were true hyphae. It is unclear why the cell type was not uniform; clearly the homozygous deletion does not display complete penetrance.

The hypoxic *HEM13* gene is not regulated by Rox1, and the *ROX1* gene is not regulated by oxygen or serum: The repression of hyphal growth by Rox1 could be direct, through repression of specific genes controlling filamentation, or indirect through repression of one or more hypoxic genes that regulate hyphal growth. To test whether Rox1 represses hypoxic genes in *C. albicans*, we analyzed the regulation of the *HEM13* gene by RT-PCR. *HEM13* encodes coproporphyrinogen III oxidase and its *S. cerevisiae* homologue is strongly repressed by the ScRox1 (ZAGOREC *et al.* 1988; KENG 1992; AMILLET *et al.* 1996). In addition, the *C. albicans HEM13* gene contains several Rox1 binding sites in its upstream region suggesting that it may be repressed by Rox1. Cells were grown aerobically or anaerobically, RNA was prepared, cDNA generated, and the *HEM13* sequences were amplified using specific probes. As a control for the efficiency of cDNA synthesis, primers that amplified a segment of the actin gene, *ACT1*, were included in the same reaction. The products of the two PCR reactions were different sizes, allowing the distinction between them. The results are presented in Figure 5A. While the accumulation of the *HEM13* mRNA was clearly repressed in the presence of oxygen (compare lane 1 with 6), no derepression occurred aerobically in the *rox1* deletion strain (compare lanes 3 and 4 with 8

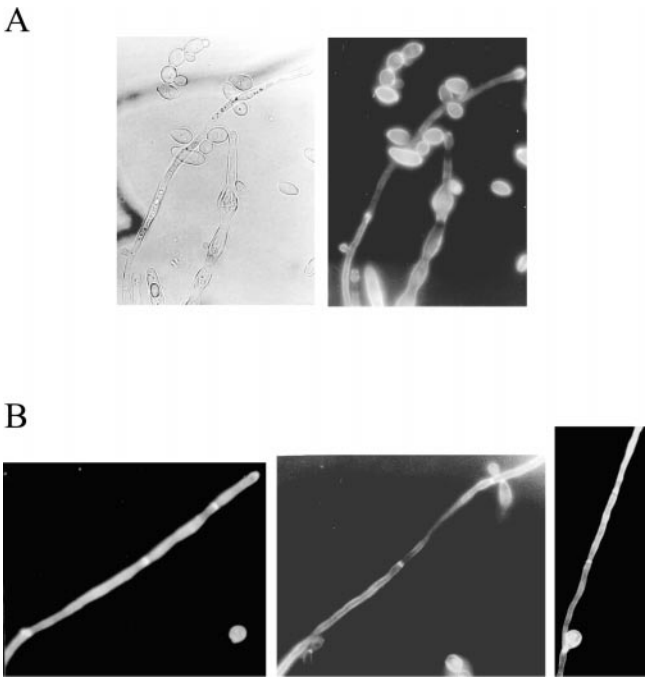

FIGURE 4.—*ROX1* deletion cultures contain true hyphae. *rox1::HIS1/rox1::HIS1* cells were stained with calcofluor (the right side of A and B) and photographed under the fluorescent microscope at ×1000.

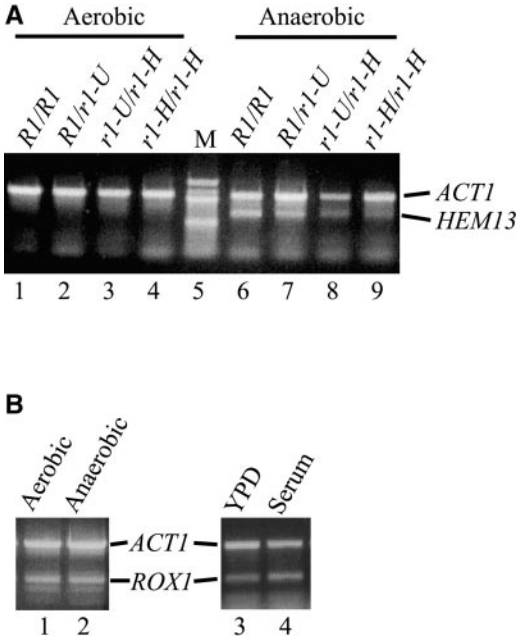

FIGURE 5.—Rox1 does not regulate the hypoxic *HEM13* gene nor are *ROX1* mRNA levels regulated by oxygen or serum. (A) RNA was prepared from wild-type cells (lanes 1 and 6), *ROX1/rox1::URA3* heterozygotes (*R1/r1-U*, lanes 2 and 7), *rox1::URA3/rox1::HIS1* double deletants (*r1-U/r1-H,* lanes 3 and 8), and *rox1::HIS1/rox1::HIS1* double deletants (*r1-H/r1-H*, lanes 4 and 9) grown either aerobically (lanes 1–4) or anaerobically (lanes 6–9). RT-PCR was carried out to determine the levels of *HEM13* and *ACT1* mRNA. The gene specific primers used resulted in fragments of different sizes for the two mRNAs. Lane 5 (M) contained a 100-bp ladder as length markers. (B) RNA was prepared from wild-type cells grown either aerobically (lanes 1, 3, and 4) or anaerobically (lane 2) in YPD (lanes 1–3) or in 10% serum (lane 4). RT-PCR was carried out amplifying *ACT1* mRNA and *ROX1* mRNA.

and 9) as would be expected if Rox1 repressed *HEM13* transcription. These results strongly suggest that Rox1 is not the hypoxic repressor in *C. albicans*.

The hypoxic response in *S. cerevisiae* cells is regulated by the transcriptional regulation of the *ScROX1* gene; the gene is transcriptionally activated in the presence of oxygen and repressed in its absence. Although the *C. albicans* gene appeared not to be the repressor of the hypoxic genes, we determined whether its transcription was regulated by oxygen using *ROX1*-specific probes for RT-PCR analyses. As seen in Figure 5B, the levels of the *ROX1* product were similar in the samples prepared from aerobic and anaerobic RNA indicating that *ROX1* expression is not regulated by oxygen.

If Rox1 were directly involved in repressing hyphal growth, its expression might be regulated by the signals that activate filamentation. One of the most potent activators is serum as seen in Figure 3. Therefore, we analyzed the accumulation of *ROX1* mRNA in cells grown with and without fetal calf serum by RT-PCR. As is evident from the results presented in Figure 5B, there was no effect of serum on *ROX1* RNA levels, suggesting that repressor function may be regulated at the level of translational or protein stability, activity, or compartmentalization.

The oxygen regulation of Rox1 hypoxic gene repression in *S. cerevisiae* is mediated by Mot3 and is Tup1dependent: The results above presented the paradox that the *C. albicans* Rox1 expressed from its own promoter appeared to regulate the hypoxic genes in *S. cerevisiae*, implying that the gene was regulated by oxygen as is the *ScROX1*, but it was not regulated by oxygen in its native cell. It seemed unlikely that the whole heme-dependent regulatory scheme that operates in *S. cerevisiae* was bent to an alternative use in *C. albicans*, especially since the *C. albicans HEM13* gene was regulated by oxygen. Therefore, we sought an alternative explanation.

Operator A, which is responsible for the bulk of the repression of the hypoxic *S. cerevisiae ANB1* gene, contains two binding sites for Rox1 and a site for a second protein, Mot3 (KASTANIOTIS *et al.* 2000). While ScRox1 is absolutely required for repression, Mot3 contributes only weakly. Mot3 and Rox1 do not bind cooperatively, but rather Mot3 appears to help in a downstream step in repression. *MOT3*, like *ScROX1,* is regulated by oxygen (C. LOWRY, personal communication; A. KASTANIOTIS and R. ZITOMER, unpublished results). We speculated that since the *C. albicans* Rox1 was a weak repressor in the heterologous host, perhaps it is much more dependent on Mot3. In that case, oxygen regulation of

ANB1 would be observed even if the *C. albicans* gene was expressed constitutively, since Mot3 concentrations are lower in aerobically grown cells. To test this possibility, we determined the ability of the intact *C. albicans ROX1* gene to repress *ANB1-lacZ* expression in a *rox1, mot3* double deletion strain (MZ22-4Δ*r1*Δ*m3*). In this strain transformed with the *S. cerevisiae ROX1* plasmid (YCp(22)*ROX1*H) the aerobic *ANB1-lacZ* expression resulted in 10.8 units of β-galactosidase compared to 126 units in the same strain transformed with the vector (YCplac22). However, when transformed with the *C. albicans* gene (YCp(22)Ca-*ROX1*), the double deletant expressed only 103 units of β-galactosidase. Thus, unlike the ScRox1, the *C. albicans* Rox1 required Mot3 for repression. Furthermore, RT-PCR analysis indicated that the *C. albicans ROX1* mRNA levels were not regulated by oxygen in *S. cerevisiae* (results not shown). Thus results demonstrate that oxygen regulation of *ANB1-lacZ* by the heterologous *ROX1* gene does not require oxygen regulation of the *C. albicans* gene in *S. cerevisiae*.

Finally, to determine whether repression by the *C. albicans* Rox1 was dependent on the general repressor Tup1 in *S. cerevisiae*, we measured the expression of the *ANB1-lacZ* fusion in the *rox1*Δ tup1Δ strain MZ22-4PC transformed with YCp(33)Ca*ROX1* or the empty vector. The presence of *CaROX1* resulted in only a 1.7-fold repression in this strain compared to the 5.5-fold repression in the congenic *TUP1* wild-type strain (Table 1). Therefore, repression by the *C. albicans* Rox1 is Tup1 dependent.

DISCUSSION

We report here the identification of a gene encoding an HMG-containing DNA binding repressor of hyphal growth from *C. albicans*. The evidence for these conclusions is as follows. The protein sequence is very similar within its HMG domain to that of the Rox1 repressor of *S. cerevisiae* and, when expressed in and purified from bacterial cells, this *C. albicans* HMG domain was capable of specific binding to DNA. The gene complemented a *S. cerevisiae ROX1* deletion to repress the expression of an hypoxic reporter gene, indicating that the protein has repressor activity. Finally, the deletion of the gene in *C. albicans* results in constitutive hyphal growth. Given this last finding, combined with the lack of effect of the deletion on the regulation of the *C. albicans HEM13* hypoxic gene, it appears inappropriate to continue to refer to this gene as *ROX1*, the homologue of the *S. cerevisiae* hypoxic repressor gene. We propose the name *RFG1*, *R*epressor of *F*ilamentous *G*rowth.

The transition from budding growth to the invasive, hyphal growth in *C. albicans* is believed to be an important aspect of the organism's pathogenicity, and a great deal of research has focused on how this transition is regulated (reviewed in Corner and Magee 1997; Kobayashi and Cutler 1998; Mitchell 1998; Brown *et al.* 2000). There appear to be two parallel signal transduction pathways that positively regulate the expression of genes required for filamentation. A mitogen-activated protein kinase cascade activates the transcriptional activator Cph1, and a cAMP-Ras pathway activates the transcriptional activator Efg1. A mutation in either results in a loss of hyphal growth induced by some stimuli, but not by others. Only the double deletion results in an almost complete loss of hyphal growth (Lo *et al.* 1997). In addition to this positive regulation, the ability of cells to undergo hyphal growth is under negative regulation. Deletion of the *TUP1* gene results in constitutive hyphal growth, perhaps in response to a third pathway (Braun and Johnson 1997, 2000). The role of the *S. cerevisiae* Tup1 protein in repression has been extensively studied. It associates with Ssn6 to form a general repression complex that is required for the repression of genes in a number of diverse regulons. The complex has no intrinsic DNA binding activity, but rather is recruited to target genes by a regulon-specific DNA binding protein. The activity or expression of each regulon-specific repressor is regulated to determine under what conditions repression occurs. Thus, for example, the hypoxic genes are repressed under aerobic conditions only because Rox1 is synthesized only when oxygen is present, and, therefore, Tup1/Ssn6 can be recruited to the hypoxic genes only under aerobic conditions. The role of Tup1 in *C. albicans* is likely to be similar; the *C. albicans TUP1* gene can complement a *TUP1* deletion in *S. cerevisiae*, and the expression of a number of genes in *C. albicans* is induced upon deletion of *TUP1* consistent with a role of a transcriptional repressor (Braun *et al.* 2000; Braun and Johnson 1997).

By analogy to *S. cerevisiae* then, we propose that Rfg1 is the regulon-specific DNA binding protein that recruits Tup1 to the filamentation genes for the following reasons. First, deletion of *RFG1* results in a similar filamentous growth phenotype as reported for the deletion of *TUP1*. However, it should be mentioned that the *tup1* double deletant grows mostly as pseudohyphal cells on rich media (Braun and Johnson 1997), while the *rfg1* double deletant contained a great deal of true hyphae. This difference may mirror the incomplete derepression of hypoxic genes seen in a *TUP1* deletion compared to a *ROX1* deletion in *S. cerevisiae* (Balasubramanian *et al.* 1993; Deckert *et al.* 1995b). Second, Rfg1 is a sequence-specific DNA binding protein. Third, Rfg1 repressed the hypoxic genes in *S. cerevisiae* and this repression required Tup1, indicating that Rfg1 can interact with a Tup1-like general repressor. Also, we demonstrated that Rfg1 function required Mot3. Mot3's role in enhancing repression by Rox1 does not involve cooperative binding, but rather helps in either the recruitment or function of the Tup1/Ssn6 complex. Mot3 is not capable of repression in the absence of Rox1, indicating that it is not capable of Tup1/Ssn6 recruitment (or function) on its own. Therefore, since Rfg1 repres-

sion of the hypoxic reporter gene in *S. cerevisiae* was Mot3 dependent, it is likely that repression was also Tup1 dependent. Thus, Rfg1 appears to fulfill many of the criteria that would be predicted for the regulon-specific DNA binding protein that recruits Tup1 to the hyphal growth genes.

It is not clear at this point how Rfg1 activity is regulated. We found that *RFG1* mRNA accumulated during hyphal growth in serum, indicating that transcription is not regulated. Regulation might occur at any subsequent step, including translation or protein function or localization. The HMG domain of Rfg1 contains extensive sequences at both the amino and carboxyl ends that are not present in ScRox1, and either of these regions could be targets of control, as could the repression domain. Alternatively, there may be other proteins involved in the repression of the filamentation genes whose synthesis or activity is regulated. Further studies are required to resolve this question.

Finally the question remains as to how the hypoxic genes of *C. albicans* are regulated. We demonstrated that *HEM13* is repressed by oxygen, strongly suggesting that a hypoxic regulon exists in this yeast. However, we have also clearly demonstrated that the Rfg1 protein is not the hypoxic repressor despite its similarity to the *S. cerevisiae* Rox1 protein, at least in the HMG domain. We cannot rule out the existence of a second gene encoding an HMG-domain repressor protein in the *C. albicans* genome, although it has not been revealed by the sequence data yet. Alternatively, these genes may be regulated by Mot3 or a novel DNA binding protein.

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