Copper-dependent transcriptional regulation by *Candida albicans* Mac1p

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We have previously shown that copper uptake and regulation in the opportunistic pathogen *Candida albicans* has some similarities to those in *Saccharomyces cerevisiae*, including the activation of the copper transporter gene *CaCTR1* under low-copper conditions by the transcription factor CaMac1p. However, in this study, further analysis has shown that the actual mechanism of regulation by CaMac1p is different from that of its *S. cerevisiae* homologue. We demonstrate for the first time, to our knowledge, that the *CaMAC1* gene is transcriptionally autoregulated in a copper-dependent manner, in contrast to *ScMAC1*, which is constitutively transcribed. We also demonstrate that the presence of one copper response element in the promoters of *CaCTR1*, *CaMAC1* and the ferric/cupric reductase gene *CaFRE7* is sufficient for normal levels of copper-responsive transcription. In contrast, two promoter elements are essential for normal levels of copper-dependent transcriptional activation by ScMac1p. CaMac1p is also involved in the regulation of the iron-responsive transcriptional repressor gene *SFU1* and the alternative oxidase gene *AOX2*. This work describes a key feature of the copper uptake system in *C. albicans* that distinguishes it from similar processes in the model yeast *S. cerevisiae*. The importance of copper uptake in the environment of the human host and the implications for the disease process are discussed.

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INTRODUCTION

Candida species are the most frequently isolated fungal pathogens in bloodstream infections and rank fourth overall among the causative organisms of these infections (Wisplinghoff *et al.*, 2004). Currently, the most common species causing candidaemia is *Candida albicans* (Pfaffer & Diekema, 2002; Tortorano *et al.*, 2004). The effect of infection is significant, and the mortality rate of *Candida* bloodstream infections was recently estimated to be 49% (Gudlaugsson *et al.*, 2003).

C. albicans infection is associated with risk factors that decrease the competence of the patient's immune system, e.g. surgery, intravascular catheterization and antibiotic treatment (Tortorano *et al.*, 2004). Approximately 30–50% of healthy people are colonized by *Candida* species, and several properties of *C. albicans* are associated with the change from commensal organism to pathogen, including the ability to grow in yeast and hyphal forms, adherence to surfaces via adhesins and resistance to anti-fungal drugs (Navarro-Garcia *et al.*, 2001). The ability of the human body to restrict the amount of free iron available to invading micro-organisms is an important defence against infection, and the ability of pathogenic organisms to

acquire iron from the human host is a well-studied virulence factor in many bacterial and fungal pathogens (Schaible & Kaufmann, 2004). In *C. albicans* the iron transporter gene *CaFTR1* has been shown to be essential for virulence in the mouse systemic model of candidiasis (Ramanan & Wang, 2000).

Iron and copper are essential nutrients for almost all living organisms and are important for a wide variety of cellular processes (reviewed by Crichton & Pierre, 2001). The redox potential of these transition metals makes them ideal as components of molecules involved in electron transfer such as cytochrome *c* oxidase, which requires copper, haem, magnesium and zinc molecules to function (Tsukihara *et al.*, 1995). Excess copper and iron in the cell can also increase production of toxic hydroxyl radicals that can damage DNA and proteins (Urbanski & Beresewicz, 2000). Metal toxicity in *C. albicans* is prevented by ATPase transporters, which remove metals from the cell (Riggle & Kumamoto, 2000; Weissman *et al.*, 2000), and Cu/Zn-containing superoxide dismutases, which detoxify superoxides produced during Fenton chemistry (Hwang *et al.*, 2002). Copper and iron homeostasis must therefore be tightly regulated to maintain these metals at the correct physiological concentrations without causing any toxic effects to the cell.

Abbreviation: CuRE, copper response element.

Copper homeostasis and iron homeostasis in *C. albicans* are intrinsically linked due to the requirement of copper for effective iron uptake (Knight *et al.*, 2002). Copper and iron are most readily available in the ferric and cupric forms but must first be reduced by ferric reductases in order to be taken up by the high-affinity copper and iron transporters *CaCTR1* and *CaFTR1* (Knight *et al.*, 2002). Previous work in our laboratory has shown that when copper levels are lowered by deletion of the copper transporter gene *CaCTR1*, high-affinity iron uptake decreases by 96 % (Marvin *et al.*, 2004). It has been demonstrated that copper-dependent oxidase activity is required for high-affinity iron uptake in *C. albicans*, suggesting the presence of a permease/oxidase complex similar to the Ftr1p/Fet3p complex of *S. cerevisiae* (Knight *et al.*, 2002; Stearman *et al.*, 1996). There are five homologues of the *FET3* gene, including *CaFET3*, which is required for growth in low-iron media, but the effects of *CaFET3* on high-affinity iron uptake have not been determined, and one or more of the other multicopper oxidases may therefore be involved in the acquisition of iron (Braun *et al.*, 2005; Eck *et al.*, 1999). In addition to the role of copper in iron homeostasis and Cu/Zn superoxide dismutases, a number of recent transcriptional profiling experiments have indicated roles for copper transport and metabolism genes in responses to alkaline pH (Bensen *et al.*, 2004), prostaglandin (Levitin & Whiteway, 2007), phagocytosis by macrophages (Lorenz *et al.*, 2004) and fluconazole resistance (Xu *et al.*, 2006). The copper transporter gene *CaCTR1* and the transcriptional activator gene *CaMAC1* have also been shown, by our laboratory and others, to affect filamentous growth (Huang *et al.*, 2006; Marvin *et al.*, 2003). Copper metabolism therefore has an important part to play in the response of *C. albicans* to the host environment, and is involved in many of the processes associated with infection.

Previous work in our laboratory identified the high-affinity copper transporter gene *CaCTR1* and demonstrated that *CaCTR1* transcription increases under copper-starvation conditions (Marvin *et al.*, 2003). We have previously shown that three sequences identical to the *S. cerevisiae* copper response elements (CuREs) are present in the promoter of *CaCTR1* and that heterologous expression of *CaCTR1* in *S. cerevisiae* requires the transcriptional activator gene *ScMAC1* (Marvin *et al.*, 2003). We have also identified the *CaMAC1* gene as a homologue of *ScMAC1*, and we have shown that transcription of *CaCTR1* requires *CaMAC1* (Marvin *et al.*, 2004).

ScMac1p regulates genes involved in the copper and iron uptake systems in response to copper levels (De Freitas *et al.*, 2004; Gross *et al.*, 2000). ScMac1p regulates its target genes by direct binding to consensus binding sites in the promoter, known as CuREs (Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). Two CuREs are required for copper-responsive transcription of *ScCTR1*, and mutation of the CuREs in the promoter of *ScFRE1* also abolishes copper-responsive transcription (Jensen *et al.*, 1998; Labbé *et al.*, 1997). One

CuRE in the *ScFRE7* promoter was sufficient for copper-responsive transcription, but this level of transcription was significantly reduced compared with a wild-type *ScFRE7* promoter containing three CuREs (Martins *et al.*, 1998). A truncated *ScCTR3* promoter of 214 bp containing just one CuRE was able to activate *lacZ* transcription twofold under low-copper conditions, but transcription was again significantly reduced compared with the eightfold activation of *lacZ* by a full-length *ScCTR3* promoter of 1116 bp (Labbé *et al.*, 1997). At least two CuREs are therefore required to produce wild-type expression levels of *ScCTR1*, *ScCTR3*, *ScFRE1* and *ScFRE7*.

The aim of this study was to identify additional genes in the CaMac1p regulon and to determine whether or not, in *C. albicans*, the CuRE sequences play a functional role in regulation by copper and CaMac1p. We show here that at least one CuRE is required for the copper-dependent regulation of *CaCTR1* and the ferric reductase gene *CaFRE7* by CaMac1p. The *CaMAC1* gene itself is also transcriptionally autoregulated in response to copper concentrations, and this involves the single CuRE in the *CaMAC1* promoter. These findings are in contrast to observations in the model organism *S. cerevisiae*, in which the *ScMAC1* gene is constitutively transcribed and at least two CuREs are required for wild-type levels of copper-dependent transcription of *ScCTR1*, *ScCTR3* and *ScFRE7* (Jensen *et al.*, 1998; Labbé *et al.*, 1997; Martins *et al.*, 1998; Zhu *et al.*, 1998). The differences in copper regulation in *S. cerevisiae* and *C. albicans* reflect the contrasting natural environments of these two organisms. Copper regulation in *C. albicans* may be important for adaptation to the human host and in the subsequent disease process.

METHODS

Strains and growth conditions. *C. albicans* strains used in this study are listed in Table 1 and were routinely cultured at 30 °C in yeast peptone media containing 2 % glucose (YPD). Synthetic defined medium (SD) was used for selection and contained 2 % (w/v) Bactoagar, 0.67 % (w/v) yeast nitrogen base without amino acids (Bio 101) and 2 % (v/v) glucose, and was supplemented with the appropriate amino acids. Arginine and histidine were added to a final concentration of 20 µg ml⁻¹, and uridine was added to a final concentration of 50 µg ml⁻¹. SD supplemented with 1 mg ml⁻¹ 5-fluoroorotic acid (5-FOA) and 50 µg uridine ml⁻¹ was used to counterselect for *CaURA3*. Minimal defined medium (MD) was used to grow cultures for RNA extraction and for β-galactosidase assays, and was based on Wickerham's medium (Wickerham, 1946) with the addition of 20 mM tri-sodium citrate (pH 4.2) and the omission of niacin, riboflavin and para-aminobenzoic acid. Copper-restricted MD also contained 50 µM bathocuproinedisulfonic acid (BCS) and 100 µM FeCl₃. Iron-restricted MD contained 50 µM bathophenanthrolinedisulfonic acid (BPS) and 100 µM CuCl₂. The *Escherichia coli* strain XL10 Gold (Stratagene) was used for all molecular cloning and was grown in Luria-Bertani (LB) medium at 37 °C. Ampicillin was used as a selective agent at a final concentration of 100 µg ml⁻¹. All liquid cultures underwent shaking at 200 r.p.m.

RNA extraction and Northern blotting. Exponentially growing cultures in MD were used for extraction of total RNA using the hot

Table 1. *C. albicans* strains used during this study

Strain	Genotype	CuREs mutated	Reference
SC5314	Clinical isolate	NA	Gillum <i>et al.</i> (1984)
BWP17	<i>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</i>	NA	Wilson <i>et al.</i> (1999)
MEM-m2	As BWP17 but <i>mac1Δ::URA3/mac1Δ::ARG4; his1::hisG/his1::hisG::HIS1</i>	NA	Marvin <i>et al.</i> (2004)
AWP1	BWP17 with <i>RPS10/RPS10::plac-poly</i>	NA	This study
AWC1	BWP17 with <i>RPS10/RPS10::plac-poly + CaCTR1</i> promoter	None	This study
AWC1.1	MEM-m2 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	None	This study
AWC2	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	CuRE 1	This study
AWC3	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	CuRE 2	This study
AWC4	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	CuRE 3	This study
AWC5	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	CuREs 1 and 2	This study
AWC6	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	CuREs 1 and 3	This study
AWC7	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	CuREs 2 and 3	This study
AWC8	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaCTR1</i> promoter	CuREs 1, 2 and 3	This study
AWF1	BWP17 with <i>RPS10/RPS10::plac-poly + CaFRE7</i> promoter	None	This study
AWF1.1	MEM-m2 with <i>RPS10/RPS10::plac-poly + CaFRE7</i> promoter	None	This study
AWF2	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaFRE7</i> promoter	CuRE 1	This study
AWF3	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaFRE7</i> promoter	CuRE 2	This study
AWF4	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaFRE7</i> promoter	CuREs 1 and 2	This study
AWM1	BWP17 with <i>RPS10/RPS10::plac-poly + CaMAC1</i> promoter	None	This study
AWM1.1	MEM-m2 with <i>RPS10/RPS10::plac-poly + CaMAC1</i> promoter	None	This study
AWM2	BWP17 with <i>RPS10/RPS10::plac-poly + mutant CaMAC1</i> promoter	CuRE 1	This study

phenol method as described elsewhere (Schmitt *et al.*, 1990). RNA was electrophoresed on a 1.5% denaturing formaldehyde gel and transferred to a nylon filter by Northern blotting overnight. The filters were hybridized overnight at 65 °C in Church Gilbert's buffer (0.5 M Na₂HPO₄, 0.5 M NaH₂PO₄, 7% SDS, 1 mM EDTA) with probes labelled with [α -³²P]dCTP using the Klenow fragment of DNA polymerase I and random hexamers as primers. Probes were constructed by PCR using the primers listed in Table 2. Filters were washed with 3 × saline sodium citrate (SSC)/0.1% (w/v) SDS and exposed to X-ray film at −80 °C. The blots were then stripped of probe by washing the filters with 2–3 changes of boiling 0.1% SDS and rinsing with 2 × SSC. The blots were then hybridized with [α -³²P]dCTP-labelled *CaACT1* probes as a loading control. The intensity of the bands on the autoradiographs was estimated using Image J densitometry analysis software (Abramoff *et al.*, 2004).

Construction of reporter plasmids and *C. albicans* strains. The promoter regions of *CaCTR1* (orf19.3646), *CaFRE7* (orf19.7077) and *CaMAC1* (19.7068) were amplified using the primers listed in Table 2. The PCR products and the reporter plasmid *plac-poly* (A. J. Brown, personal communication) were digested with either *Sall* and *XmaI* (*CaCTR1*) or *XhoI* and *XmaI* (*CaFRE7* and *CaMAC1*). The promoter fragments were ligated to the digested plasmid and transformed into XL10 Gold *E. coli* using the calcium chloride method (Mandel & Higa, 1970). Recombinant plasmid DNA was isolated using the Qiagen Miniprep extraction kit and each plasmid was transformed into BWP17 and FOA-treated MEM-m2 (*mac1Δ/mac1Δ*) using the lithium acetate method, as described elsewhere (Gietz & Woods, 2002). The *plac-poly* plasmid integrates into the genome at *CaRPS10* in the same way as *Cip10* (Murad *et al.*, 2000), and integration of one copy of all the plasmids was confirmed by Southern blotting using a fragment of the *plac-poly bla* gene as a probe (data not shown).

Site-directed mutagenesis. The existing reporter plasmids containing the promoters of *CaCTR1* (AWC1), *CaFRE7* (AWF1) and *CaMAC1* (AWM1) were used as templates to generate reporter plasmids containing mutated CuRE sequences. The PCR-based

mutagenesis strategy was modelled on the QuikChange XL site-directed mutagenesis kit from Stratagene. Mutagenesis primers (Table 2) were designed to incorporate mutations of two or three bases in one of the CuRE sequences to alter it from the consensus sequence TTTGC(T/G)C(A/G). Previous work has shown that any deviation from this consensus sequence abolishes binding of ScMac1p and results in the loss of copper-dependent regulation in β -galactosidase reporter assays (Jensen *et al.*, 1998; Labbé *et al.*, 1997; Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). The mutagenesis primers all had a melting temperature (T_m) of at least 68 °C, and each pair of primers (forward and reverse) was complementary, and in opposite orientations. Pairs of primers were used to amplify both strands of the template reporter plasmid (AWC1, AWF1 or AWM1) using BIO-X-ACT Long DNA polymerase (BioLine), and the high T_m of the primers ensured that the primers containing the mutations remained annealed to the template throughout the extension step. Following amplification, the methylated template DNA was digested into small fragments with *DpnI* and the newly synthesized DNA was transformed into XL10 Gold *E. coli*. The mutations were confirmed by sequencing and the mutant plasmid was transformed into BWP17 (wild-type). Integration of one copy of the reporter plasmid was confirmed by Southern blotting (data not shown).

In the *CaCTR1* promoter, CuRE 1 (located 397 bp upstream of the *CaCTR1* ORF) was mutated to give strain AWC2, CuRE 2 (located at −275 bp) was mutated to give strain AWC3 and CuRE 3 (at −237 bp) was mutated to give strain AWC4. The single mutants were then used as templates to generate strains AWC 5–8 with the mutations in the CuREs indicated in Table 1. Table 1 also indicates the mutations made in the promoters of *CaFRE7* and *CaMAC1* to generate strains AWF2–4 and AWM2.

Assay for β -galactosidase activity. Cultures of reporter strains were grown to exponential phase in 5 ml MD with different concentrations of copper, and were assayed for β -galactosidase activity by the permeabilized cell method, as described elsewhere (Rupp, 2002).

Table 2. Oligonucleotides used in this study

The positions of CuREs are shown in italic type and mutated bases in underlined type.

Primer name	Sequence (5'–3')	Use of primer
ACT1 F	GGTAGACCAAGACATCAAGG	<i>CaACT1</i> probe
ACT1 R	GAACCACCAATCCAGACAGAG	<i>CaACT1</i> probe
AOX2 F	TCCAGCTTTCCATCAACCAA	<i>AOX2</i> probe
AOX2 R	GTGAACCAACTTGGTTTACCA	<i>AOX2</i> probe
CaFRE7 F	GCCTACATGAAGTACAAGC	<i>CaFRE7</i> probe
CaFRE7 R	GGACCACGTGAATGACTG	<i>CaFRE7</i> probe
OrfMAC1 F	CTAGCAGTAGAACGTTTCGTG	<i>CaMAC1</i> probe
OrfMAC1 R	GCGTATCTCGATTGTTGACC	<i>CaMAC1</i> probe
SFU1 F	GTCCTGTAAATCTTAAACGG	<i>SFU1</i> probe
SFU1 R	GATGATTGCATTGGTGAATG	<i>SFU1</i> probe
<i>CaCTR1</i> 776 F	GATCGTCGACGTCCCTACAGTAAACAAGGC	<i>CaCTR1</i> reporter
<i>CaCTR1</i> 776 R	GATCCCCGGAATGTAAGGTTAAATGTGGTGTG	<i>CaCTR1</i> reporter
<i>CaFRE7</i> 796 F	GATCCTCGAGGATTTCTTGCATTGTTTCGG	<i>CaFRE7</i> reporter
<i>CaFRE7</i> 796 R	GATCCCCGGGTTCAATGGTTTCAAGAAAATTCAAG	<i>CaFRE7</i> reporter
<i>CaMAC1</i> 683 F	GATCCTCGAGCGGCATTGTTAAGACTGAAATGGG	<i>CaMAC1</i> reporter
<i>CaMAC1</i> 683 R	GATCCCCGGGTCCTTATTCAGTCTTGCTTTTGGAGG	<i>CaMAC1</i> reporter
<i>CaCTR1-1</i> F	GGATTGGTAAGAGATTGATGAGCATATGCAAAATTTCAATTACC	<i>CaCTR1</i> CuRE 1 mutation
<i>CaCTR1-1</i> R	GGTAATTGAAAAATTTGCATATGCTCATCAATCTCTTACCAATCC	<i>CaCTR1</i> CuRE 1 mutation
<i>CaCTR1-2</i> F	GGGTCAAAGAATAATTTGTTAACTTGAGATTAAATTATTAGGACTTAGTC	<i>CaCTR1</i> CuRE 2 mutation
<i>CaCTR1-2</i> R	GACTAAGTCCTAATAATTAAATCTCAAGTGTTAAACAAATATTCTTTGACCC	<i>CaCTR1</i> CuRE 2 mutation
<i>CaCTR1-3</i> F	GGAAAGAAAAGAAAATAACGCGTAAAATAAAAAGGTATTTAAAGGG	<i>CaCTR1</i> CuRE 3 mutation
<i>CaCTR1-3</i> R	CCCTTTCCCTACCTTTTATTTTACGCGTATTTTCTTTCTTTCTTTCC	<i>CaCTR1</i> CuRE 3 mutation
<i>CaFRE7-1</i> F	CCCCGAAACAGTAATAATTACAAAGTAATAAGCTTAAAAATTTTACATTATAATTACG	<i>CaFRE7</i> CuRE 1 mutation
<i>CaFRE7-1</i> R	CGTAATTATAATGTGAAAAATTTAAGCTTATTACTTTGTAATTATTACTGTTTCGGGG	<i>CaFRE7</i> CuRE 1 mutation
<i>CaFRE7-2</i> F	GTGCTGCAAAAATGGCCAAATTATTGGAACCCCG	<i>CaFRE7</i> CuRE 2 mutation
<i>CaFRE7-2</i> R	CGGGGTTCCAATAATTTGGCCATTTTGCGACAC	<i>CaFRE7</i> CuRE 2 mutation
<i>CaMAC1-1</i> F	CCATTCCCAATCGGGAATGATAAATTTGGCCATCCCGCTTC	<i>CaMAC1</i> CuRE 1 mutation
<i>CaMAC1-1</i> R	GAAGCGGGATGGCCAAATTTATCATTTCCCGATTGGGAATGG	<i>CaMAC1</i> CuRE 1 mutation

RESULTS

Identification of genes with promoters containing putative CaMac1p binding sites

The promoters of all ORFs in assembly 19 of the *C. albicans* genome sequence were searched using the Ca19 ORF/Gene 1000 bp upstream motif search tool on the web pages of the *Candida albicans* research lab at the National Research Council Canada (<http://www.candida.bri.nrc.ca/candida>). Searches for the consensus binding site sequence in both orientations were carried out, and 1120 ORFs contained one or more CuREs in either the 5'→3' direction or the 3'→5' direction. The results of both searches were collated and 20 ORFs were identified that contained two or more putative binding sites in their promoters (Table 3). The genes identified in the search have a wide variety of functions, and 25% are involved in copper and iron metabolism. They include the copper transporter gene *CaCTR1*, the iron permease gene *CaFTR2*, two ferric reductase-like genes (*CaFRE3* and *CaFRE7*) and the iron-responsive transcriptional repressor gene *SFU1*. We have demonstrated previously that *CaCTR1* (orf19.3646) is regulated by copper and CaMac1p (Marvin *et al.*, 2004).

It is interesting to note that the upstream motif search revealed that the *CaMAC1* promoter itself contains a CuRE sequence 167 bp upstream of the ORF.

Genes were selected for further analysis based on either their functions or the positions of their putative binding sites. It is possible that CaMac1p regulates genes that are involved in iron and copper metabolism, and three genes were selected for further analysis based on their putative functions in these processes (*CaFRE3*, *CaFRE7* and *SFU1*). The *AOX2* and orf19.6238 genes were chosen for further analysis because they have two CuREs within their promoters that are less than 300 bp from the translation start site, a position that is typical of transcription factor binding sites in the promoters of *C. albicans* genes (Tirosh *et al.*, 2007). The putative CuREs in the promoters of *CaFRE3* and *CaFRE7* are also located in this region of the promoter, but the CuRE sequences in the *SFU1* promoter are outside the normal locations for transcription factor binding sites. Orf19.7078 was also analysed because it is divergently transcribed from *CaFRE7* and the two genes share their promoter and the two CuREs within it, although the CuRE sequences are approximately 900 bp upstream of orf19.7078.

Table 3. Results of upstream motif search for two putative CaMac1p binding sites using the Ca19 ORF/Gene 1000 bp upstream motif search tool at www.candida.bri.nrc.ca/candida

Gene names and functions were identified from www.candidagenome.org.

orf	Gene name	Motif		Distance between motifs (bp)	Gene function
		YGMGCAAA	TTTGCKCR		
19.333	<i>CaFCY2</i>	−995 −292		503	Purine/cytosine permease
19.681	<i>HAP43</i>	−928 −578		350	Transcription factor. Similar to AP-1 from <i>Neurospora crassa</i>
19.1971		−699 −592		107	Unknown function
19.1270	<i>CaFRE3</i>	−531	−801	270	Ferric reductase
19.3282		−978 −465		513	Unknown function
19.3303		−808	−457	351	Carboxy-methyl transferase
19.3646	<i>CaCTR1</i>	−275	−397 −237	122, 38	High-affinity copper permease
19.4146	<i>CaSMD3</i>	−796 −187	−969	173, 609	Core snRNP protein for nucleic acid metabolism
19.4773	<i>AOX2</i>	−776 −177 −149		599, 28	Alternative oxidase for respiratory pathway
19.5599	<i>MDL2</i>	−727	−675	52	ATP-binding membrane transporter
19.4855	<i>BUD31</i>	−794 −76		718	Bud site selection
19.4869	<i>SFU1</i>	−823	−576	247	GATA-type transcriptional repressor
19.5195		−180	−191	Overlapping	Uridine-monophosphate kinase
19.6007		−714	−601	113	Fatty acid elongation
19.6016		−102	−966	864	Vesicle transport protein
19.6238		−277 −122		155	Unknown function
19.7077	<i>CaFRE7</i>	−177	−132	45	Ferric reductase
19.7078		−937	−892	45	Unknown function; shares promoter with <i>CaFRE7</i>
19.7231	<i>CaFTR2</i>	−501 −9		492	High-affinity iron permease
19.7625	<i>PGA1</i>	−309	−950	641	Unknown function

Previous analysis of the putative *C. albicans* ferric reductases in our laboratory has placed *CaFRE3* in a group of ferric reductases that are most similar to *ScFRE1*–6, and has also shown that *CaFRE7* is the *C. albicans* ferric reductase gene that is most similar to *ScFRE7* (unpublished data). *SFU1* encodes a GATA-type transcriptional repressor that represses several genes with functions in iron uptake, including the ferric reductases *FRE1* and *FRE2*, iron permeases and multicopper oxidases (Lan *et al.*, 2004). orf19.6238 has no known function, but *AOX2* encodes an alternative oxidase involved in cyanide-resistant respiration, and accepts electrons from the electron transfer chain (Huh & Kang, 2001).

Northern blot analysis of genes containing two CuRE sequences in their promoters

Total RNA was extracted from cultures of BWP17 (wild-type) and *mac1Δ/mac1Δ* strains grown in copper-restricted MD media with and without additional 100 μM CuCl₂ or in iron-restricted MD media with and without additional 100 μM FeCl₃. Northern blots were hybridized with [α -³²P]-labelled fragments of the ORFs of *CaFRE7*, *CaMAC1*, *SFU1*, *AOX2*, *CaFRE3*, orf19.6238 and orf19.7078. The blots were also hybridized with *CaACT1* as a loading control (see Methods and Table 2 for probe sequences).

Transcripts for *CaFRE7* were only detected at low copper levels in the wild-type (BWP17), and no transcript was visible in the absence of *CaMAC1* (Fig. 1). This indicates that

CaFRE7 transcription is activated by CaMac1p in response to copper starvation, and this evidence suggests that *CaFRE7* may be the functional homologue of *ScFRE7* (Martins *et al.*, 1998). We also demonstrated that *CaMAC1* transcription in the wild-type is increased approximately fivefold in response to low copper levels and confirmed that no transcript is detected in the *mac1Δ/mac1Δ* mutant (Fig. 1). There was no difference in the levels of *AOX2* transcript under different copper conditions in the wild-type, and no transcript could be detected in the *mac1Δ/mac1Δ* strain (Fig. 1).

Transcription of *SFU1* in the wild-type was not significantly affected by low-copper conditions, but transcription was repressed 11-fold under low-iron conditions (Fig. 2). *SFU1* transcripts in the *mac1Δ/mac1Δ* mutant were more abundant under all conditions tested, but regulation of *SFU1* transcription by copper and iron levels was maintained. Transcription of *SFU1* in the *mac1Δ/mac1Δ* mutant was repressed threefold under low-copper conditions and sixfold under low-iron conditions (Fig. 2). A transcript for *CaFRE3* could not be detected, and no change in the expression levels of orf19.6238 or orf19.7078 was observed in response to copper, iron or *CaMAC1* (data not shown).

Copper-responsive transcriptional regulation of *CaCTR1*, *CaFRE7* and *CaMAC1* by CaMac1p

Northern blot analysis indicated that *CaCTR1* and *CaFRE7* are regulated by CaMac1p in a copper-dependent manner,

(b)

Gene	BWP17 expression levels		<i>mac1Δ/mac1Δ</i> expression levels	
	+ Cu	- Cu	+ Cu	- Cu
<i>CaFRE7</i>	0.01	0.84	0.01	0.01
<i>CaMAC1</i>	0.05	0.28	0.03	0.03
<i>AOX2</i>	0.58	0.76	0.01	0.02

Fig. 1. Northern blot analysis of genes containing CuREs in their promoters. (a) Total RNA was extracted from *C. albicans* strains BWP17 (wild-type) and MEM-m2 (*mac1Δ/mac1Δ*) growing at exponential phase in copper-restricted MD medium with 100 μ M CuCl_2 (lanes 1 and 3) or 0 μ M CuCl_2 (lanes 2 and 4). Northern blots were hybridized with [α - 32 P]-labelled probes for *CaFRE7*, *CaMAC1* and *AOX2* (see Table 2 for primers used in the construction of probes). Each blot was also hybridized with an [α - 32 P]-labelled *CaACT1* probe as a loading control. The blots shown are representative examples of at least three repetitions for each probe used. (b) Expression levels of the genes in Fig. 1(a) are shown as a proportion of the expression level of the *CaACT1* control.

and reporter plasmids containing the promoters of these genes were constructed in order to investigate this. The CuRE sequences in these promoters were mutated to determine their role in copper-responsive regulation by CaMac1p. The copper-dependent transcription of *CaMAC1* and the presence of a CuRE sequence in the *CaMAC1* promoter suggested that *CaMAC1* might be autoregulated in response to copper. Therefore, a *CaMAC1* promoter-*lacZ* reporter plasmid was also constructed to investigate whether or not this was the case.

β -Galactosidase reporter constructs containing the promoters of *CaCTR1*, *CaFRE7* and *CaMAC1* were transformed into BWP17 (wild-type) and *mac1Δ/mac1Δ*. Integration of one copy of the plasmid into the genome at *CaRPS10* was confirmed by PCR and Southern blotting

(b)

	<i>SFU1</i> expression levels in BWP17	<i>SFU1</i> expression levels in <i>mac1Δ/mac1Δ</i>
+ Cu	0.11	0.77
- Cu	0.07	0.23
+ Fe	0.11	0.94
- Fe	0.01	0.14

Fig. 2. Northern blot analysis of *SFU1*. (a) Total RNA was extracted from *C. albicans* strains BWP17 (wild-type) and MEM-m2 (*mac1Δ/mac1Δ*) growing at exponential phase in copper-restricted MD medium with 100 μ M CuCl_2 (lanes 1 and 5) or 0 μ M CuCl_2 (lanes 2 and 6), and in iron-restricted MD medium with 100 μ M FeCl_3 (lanes 3 and 7) or 0 μ M FeCl_3 (lanes 4 and 8). Northern blots were hybridized with [α - 32 P]-labelled probe for *SFU1* and *CaACT1* (see Table 2 for primers used in the construction of probes). The blot shown is a representative example of at least three repetitions. (b) Expression levels of *SFU1* are shown as a proportion of the expression level of the *CaACT1* control.

(data not shown). The reporter constructs with *CaCTR1*, *CaFRE7* and *CaMAC1* promoters showed an increase in β -galactosidase activity of 3.1-fold, 4.4-fold and 2.8-fold, respectively, under low-copper conditions in BWP17 (Fig. 3). In the *mac1Δ/mac1Δ* strain, all constructs showed lower levels of activity than in the wild-type, and copper-dependent regulation of activity was abolished (Fig. 3). This data shows that transcription of *CaCTR1*, *CaFRE7* and *CaMAC1* is activated by CaMac1p in response to copper starvation.

The putative CuREs in the *CaCTR1* promoter have a role in copper-dependent regulation by CaMac1p

The site-directed mutagenesis strategy used to mutate CuRE sequences in the *CaCTR1* promoter-*lacZ* reporter plasmids is described in Methods. These mutations included single mutations in CuRE 1, 2 or 3 and all possible combinations of two mutated CuREs. A mutant was also constructed that contained mutations in all three CuREs. The mutated plasmids were all transformed into the wild-type *C. albicans* strain BWP17 to generate seven reporter strains (AWC2–8, see Table 1). The reporter strains were grown in copper-restricted MD media

Fig. 3. β -Galactosidase activity of wild-type promoter constructs. Assays for β -galactosidase activity were performed on strains growing at exponential phase in copper-restricted MD containing 100 μ M CuCl_2 (black bars) or 0 μ M CuCl_2 (white bars). Results are shown for BWP17 (wild-type) and MEM-m2 (*mac1Δ/mac1Δ*), each transformed with *plac-poly*, *CaCTR1* promoter–*lacZ* fusion, *CaFRE7* promoter–*lacZ* fusion and *CaMAC1* promoter–*lacZ* fusion. The means of three independent experiments are shown; error bars show 1SD.

containing high and low levels of copper, and β -galactosidase activity was measured as described in Methods.

When only CuRE 1 was functioning (AWC7), there was a higher level of expression compared with the wild-type *CaCTR1* promoter (Fig. 4). The amount of β -galactosidase activity increased under high-copper conditions from 1.68

units μl^{-1} for the wild-type promoter to 2.35 units μl^{-1} for AWC7, and from 5.27 to 10.93 units μl^{-1} under low-copper conditions. When only CuRE 2 was functioning (AWC6), there was a decrease in β -galactosidase activity compared with the wild-type promoter (Fig. 4). Activity decreased by 23 % under high-copper and by 60 % under low-copper conditions. When only CuRE 3 was functioning (AWC5), β -galactosidase activity decreased by 65–85 %

Fig. 4. β -Galactosidase activity of mutant *CaCTR1* promoter–*lacZ* fusions. The *CaCTR1* promoter was mutated as described in Methods with combinations of one, two or three mutations of the CuRE sequences. A schematic representation to the left of the graph of the *CaCTR1* promoter of each strain shows wild-type CuRE sequences as white boxes and mutated CuRE sequences as grey boxes. CuRE 1 is distal to the *CaCTR1* ORF and CuRE 3 is proximal to the ORF. All reporter plasmids containing the *CaCTR1* promoter were transformed into BWP17 (wild-type) to construct *C. albicans* strains AWC1–8, which are described in further detail in Methods. Assays for β -galactosidase activity were performed on strains growing at exponential phase in copper-restricted MD containing 100 μ M CuCl_2 (black bars) or 0 μ M CuCl_2 (white bars). The means of three independent experiments are shown; error bars show 1SD.

compared with the wild-type and there was no difference in activity in response to copper levels (Fig. 4). In a mutant without any functioning CuRE sequences (AWC 8), β -galactosidase activity was also reduced by 65–85 % compared with the wild-type and was not copper-responsive (Fig. 4). This level of activity was similar to the activity of the *CaCTR1* promoter–*lacZ* reporter in a *mac1Δ/mac1Δ* strain (Fig. 3).

When CuREs 1 and 2 were functioning (AWC4), β -galactosidase activity was not significantly different from that with the wild-type promoter (Fig. 4). When CuREs 1 and 3 were functioning (AWC3), β -galactosidase activity was not significantly affected by high copper levels, but activity did show an increase of 40 % at low copper concentrations compared with the wild-type promoter (Fig. 4). When CuREs 2 and 3 were functioning (AWC2), there was a decrease of ~70 % in β -galactosidase activity under both high- and low-copper conditions (Fig. 4). Although the overall level of expression was lower in this construct, β -galactosidase activity was still copper-regulated and was higher under low-copper conditions. This evidence demonstrates that one copy of CuRE 1 or 2 in the *CaCTR1* promoter is sufficient but necessary for copper-responsive activation of the *lacZ* gene. A reporter construct containing only CuRE 1 in the promoter demonstrated levels of copper-responsive expression that were at least equal to those of the wild-type promoter. The CuRE sequences do not contribute equally to the activation of *lacZ*; CuRE 1 had the most effect on transcription and CuRE 3 the least effect. This indicates that a complex

interaction is taking place between the CaMac1 protein and the CuRE sequences that is influenced by different combinations of CuREs.

One CuRE is necessary and sufficient for activation of *CaFRE7* and *CaMAC1* under low-copper conditions

The *CaFRE7* promoter–*lacZ* reporter construct was used as a template for site-directed mutagenesis of the CuRE sequences to construct strains AWF2–4, as described in Methods. Reporter strains were grown under high- and low-copper conditions, and β -galactosidase activity was measured. Mutation of CuRE 1 in the *CaFRE7* promoter (AWF2) caused a 1.8-fold increase in β -galactosidase activity at low copper levels compared with the wild-type promoter (Fig. 5). This was accompanied by a decrease in activity of 37 % at high copper levels, and resembled the alteration in β -galactosidase activity when CuRE 2 in the *CaCTR1* promoter was mutated (Fig. 4). In contrast, mutation of CuRE 2 in the *CaFRE7* promoter (AWF3) resulted in a decrease in β -galactosidase activity of 96 % at high copper levels, but did not significantly affect activity under low-copper conditions (Fig. 5). Mutation of both CuREs (AWF4) reduced β -galactosidase activity to a level similar to that of the *CaFRE7* construct in a *mac1Δ/mac1Δ* background and also abolished copper regulation. One of the two CuREs in the *CaFRE7* promoter is therefore necessary and sufficient for copper-responsive β -galactosidase activity equal to that of the wild-type promoter.

Fig. 5. β -Galactosidase activity of mutant *CaFRE7* and *CaMAC1* promoter–*lacZ* fusions. The *CaFRE7* and *CaMAC1* promoters were mutated as described in Methods with either one or two CuREs mutated. A schematic representation to the left of the graph of the *CaFRE7* and *CaMAC1* promoters shows wild-type CuRE sequences as white boxes and mutated CuRE sequences as grey boxes. CuRE 1 is distal to the *CaFRE7* ORF and CuRE 2 is proximal to the ORF. AWF1–4 are constructs containing the *CaFRE7* promoter and AWM1–2 are constructs containing the *CaMAC1* promoter. All mutant reporter plasmids were transformed into BWP17 (wild-type). Assays for β -galactosidase activity were performed on strains growing at exponential phase in copper-restricted MD containing 100 μ M CuCl₂ (black bars) or 0 μ M CuCl₂ (white bars). The means of three independent experiments are shown; error bars show 1SD.

Site-directed mutagenesis of the CuRE in the *CaMAC1* promoter (AWM2) decreased β -galactosidase activity by 70% under low-copper conditions compared with the wild-type *CaMAC1* promoter (Fig. 5). There was no significant difference in β -galactosidase activity under high- or low-copper conditions (Fig. 5), indicating that the CuRE is involved in copper-responsive regulation of *CaMAC1* and demonstrating for the first time, to our knowledge, that *CaMAC1* is transcriptionally autoregulated.

DISCUSSION

This study has demonstrated that *CaCTR1*, *CaFRE7* and *CaMAC1* are regulated in a copper-responsive manner in the presence of the *CaMAC1* gene. The presence of at least one CuRE sequence in the promoters of these genes is required for maximal expression levels and for copper-responsive regulation. This evidence supports the hypothesis that CaMac1p binds to CuRE sequences in target promoters when copper is scarce to activate transcription of these components of the *C. albicans* copper uptake system.

The finding that one CuRE is sufficient and necessary for activation by CaMac1p shows a key difference between *C. albicans* and *S. cerevisiae*, because ScMac1p has been shown to require two CuREs to activate target genes (Martins *et al.*, 1998). Transcriptional autoregulation of the *CaMAC1* gene in response to copper levels is in contrast to the regulation of *ScMAC1*, which is constitutively transcribed (Zhu *et al.*, 1998). The copper-responsive transcription factors Ace1p and Amt1p from *S. cerevisiae* and *Candida glabrata* both show variation in the number of binding sites required for activation activity, depending on the target gene (Liu & Thiele, 1997). This may also be the case for CaMac1p, and analysis of the promoters of additional genes regulated by CaMac1p will determine whether or not this is the case.

The involvement of CaMac1p in the regulation of *AOX2* and *SFU1* suggests that gene activation by CaMac1p may also affect other aspects of metabolism in addition to copper uptake. The regulation of *AOX2* is different from the regulation of alternative oxidase genes from other fungi, where the transcription of alternative oxidases increases under low-copper conditions because a shortage of copper impairs the activity of copper-containing respiratory enzymes such as cytochrome *c* oxidase (Borghouts *et al.*, 2001; Downie & Garland, 1973).

Transcription of the iron-responsive transcriptional repressor gene *SFU1* is repressed under iron-starvation conditions in both the wild-type and the *mac1Δ/mac1Δ* mutant, and this results in derepression of iron uptake genes regulated by Sfu1p, such as the siderophore transporter *CaARN1* and the ferric reductases *CFL1* and *CFL2* (Lan *et al.*, 2004). *SFU1* transcription shows a small decrease under low-copper conditions in the wild-type and a more significant decrease under low-copper conditions in the

mac1Δ/mac1Δ mutant. Copper is essential for iron uptake in *C. albicans*, and a copper-limiting environment may lead to a decrease in the ability of the cell to take up iron and therefore over time this environment may also become iron-limiting and cause repression of *SFU1* transcription. The *mac1Δ/mac1Δ* mutant is able to take up less copper than the wild-type strain due to the decreased level of *CaCTR1* transcription and is likely to be more severely starved of copper and iron than the wild-type. Repression of *SFU1* transcription in low-copper medium is therefore more pronounced in the mutant. The higher overall level of *SFU1* transcription in the *mac1Δ/mac1Δ* mutant does not, however, appear to be a response to copper or iron levels, as copper or iron starvation would likely result in decreased transcription of *SFU1*.

CaMac1p is unlikely to be directly activating transcription of *SFU1* and *AOX2* in a manner similar to the copper-responsive regulation of *CaCTR1*, *CaFRE7* and *CaMAC1*. CaMac1p could therefore be activating an intermediate regulator or acting as a co-activator or co-repressor to cause an effect on transcription levels that is not copper-dependent. Irrespective of the mechanism by which CaMac1p is affecting transcription, the influence of copper-responsive CaMac1p on the expression of the iron-responsive transcription factor gene *SFU1* is an important link between the regulation of the high-affinity iron and copper uptake systems, which both show major differences in regulation from their homologous *S. cerevisiae* systems.

A number of the copper metabolism genes investigated in this study have also been implicated in the response of *C. albicans* to host factors, including engulfment by macrophages and the secretion of prostaglandin. Transcript profiling studies have recently shown that expression of *CaFRE7*, *CaMAC1*, *CaCTR1* and the ferric reductase-like gene orf19.6139 is repressed in response to prostaglandin (Levitin & Whiteway, 2007). Prostaglandin is produced by endothelial cells and is stimulated by contact with *C. albicans* (Filler *et al.*, 1994). During the preparation of this manuscript, further analysis of *CaFRE7* demonstrated that prostaglandin-mediated repression only occurs in the yeast growth phase and that a ScMac1p binding site sequence between 120 and 134 bp upstream of the ORF, designated CuRE 2 in the present study, is involved in the regulation of *CaFRE7* expression in YPD medium (Levitin & Whiteway, 2007). However, the effect of copper levels and the role of the second putative CaMac1p binding site at position –177 bp were not investigated in the study of Levitin & Whiteway. Three copper metabolism genes (*CaFRE7*, *CaCTR1* and orf19.6139) are also upregulated during phagocytosis by macrophages (Lorenz *et al.*, 2004). The change in expression of copper metabolism genes in response to the host immune system indicates that copper metabolism may have a more significant role when *C. albicans* is attacked by macrophages than when the organism comes into contact with prostaglandin-producing endothelial cells.

This study demonstrates that CaMac1p is autoregulated and can also regulate copper metabolism genes via one CuRE sequence in their promoters. This evidence shows that copper-dependent regulation by CaMac1p in the human pathogen *C. albicans* is different from copper-dependent regulation by ScMac1p in the model yeast *S. cerevisiae*. The transcriptional autoregulation of the *CaMAC1* gene may be an adaptation to the host environment, and there are several possible advantages to this system of regulation. *C. albicans* has a high tolerance to copper, suggesting that some niches colonized by the organism may have very high copper concentrations (Riggle & Kumamoto, 2000; Weissman *et al.*, 2000). As *CaMAC1* is only expressed under conditions of copper starvation, the basal level of transcriptional activation of *CaCTR1*, *CaFRE7* and other genes in the CaMac1p regulon would be very low in high-copper environments. This would prevent unnecessary transcription and translation of genes when they are not required, and would conserve energy and nutrients, as well as preventing uptake of potentially toxic levels of copper. In niches where the availability of copper is low, transcription of the *CaMAC1* gene would be induced, and this would increase the number of CaMac1p molecules available to activate target genes such as *CaCTR1* and help to alleviate copper starvation. Copper-dependent regulation of *CaMAC1* transcription may therefore be an adaptation to help *C. albicans* to survive in both high- and low-copper environments, and this could be a competitive advantage in the human host.

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