Copper-responsive transcriptional regulation in *Candida albicans*

Thesis submitted for the degree of Doctor of Philosophy
University of Leicester

by

Alexandra Woodacre BSc (University of Leeds)

October 2007

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Table of Contents

Chapter 1
General Introduction

Chapter 2

Materials and Methods

Chapter 3

Analysis of putative CaMac1p binding sites in the *CaCTR1* promoter

Chapter 4

Identification of genes with promoters containing putative CaMac1p binding sites

Chapter 5

Analysis of putative CaMac1p binding sites in the promoter of *CaFRE7*

Chapter 6

Transcriptional analysis of the transcriptional activator gene *CaMAC1*

List of Figures

List of Tables

Acknowledgements

I would like to thank Annette for her unfailing support, encouragement and advice. She has always been there for me when it counted, through good and bad, and has taught me so much. Thank you to Rose – you have been a pleasure to work with and I think we make a great team. I would also like to thank the students who have worked with me including Tom, Mark, Alex and David, but especially to Bobby Kakavitsas for his help in constructing the reporter plasmids used in Chapter 5. I am very grateful to Julie Morrissey, Julian Ketley and the force of nature that is Marcus Marvin for helpful discussions about my work and ideas for the project.

There are too many people to mention here but I would like to thank each and every member of both my old and new labs G2/G7 and 121 for their friendship during the last four years. Keith, Liz, Lindsay, Gary and Rachel in the kitchen and the incomparable Pat Loft have also been fabulous and have helped me out so much with media, tips, glassware, pipettes etc. etc.

I want to thank my family, and especially my mum, for having unfailing love and belief in me. Thank you also to some very special friends - Morag, Alex, Ruth, Rich, Miranda, Geetha, Stu, Andy, Vicky, Elaine, Nic W, Nic B, Rach and Liz.

Thank you also to Annette, Ruth, Marie and all the members of the 5th Leicester Guides for giving me something else to think about apart from science!

Rob – thank you for everything. You make bad things good and good things even better. I am looking forward to being Dr. Woodacre, but not as much as I am looking forward to being Mrs Mason. I love you.

Abstract

Candida albicans is a commensal fungus of humans and can cause opportunistic infections if the host immune system is compromised. *C. albicans* infections can range from relatively minor subcutaneous infections to life-threatening systemic candidiasis. Copper is an essential nutrient for almost all organisms, and the copper-containing protein superoxide dismutase is required for the virulence of *C. albicans* in a mouse model. Previous work in our laboratory has shown that copper uptake and regulation in *C. albicans* has some similarities to *Saccharomyces cerevisiae*, including the activation of the copper transporter gene *CaCTR1* in low copper conditions by the transcription factor CaMac1p. However, further analysis in this study has demonstrated that the actual mechanism of regulation by CaMac1p is different from that of *S. cerevisiae* Mac1p.

This thesis demonstrates for the first time that the *CaMAC1* gene is transcriptionally autoregulated in a copper-dependent manner. This is in contrast to the *S. cerevisiae MAC1* homologue, which is constitutively transcribed. The presence of one binding site for CaMac1p in the promoters of *CaCTR1*, *CaMAC1* and the ferric/cupric reductase gene *CaFRE7* is sufficient for copper-responsive regulation. In contrast, two promoter elements are essential for normal levels of copper-dependent activation by *S. cerevisiae* Mac1p. CaMac1p is also involved in the regulation of the iron-responsive transcriptional repressor gene *SFU1* and the alternative oxidase gene *AOX2*.

This work describes key features of the copper uptake system in the human pathogen *C. albicans* that distinguishes it from similar processes in the model yeast *S. cerevisiae*. Transcriptional autoregulation of the *CaMAC1* gene could enable *C. albicans* to respond more precisely to environmental changes, conferring an adaptation to the human host that may be an advantage in the disease process.

Abbreviations

BCS	Bathocuproinedisulfonic acid
BPS	Bathophenanthrolinedisulfonic acid
bp	base pair
BSA	Bovine serum albumin
CuRE	Copper response element
DNA	Deoxyribonucleic acid
DEPC	Di-ethyl-pyrocarbonate
EDTA	Ethylenediaminotetra acetic acid
5-FOA	5-Fluoroorotic acid
LA	Luria agar
LB	Luria broth
g	Grams
HEPES	N-(2-hydroxyethyl) piperazine N'-(2-ethanesulfonic acid)
kb	Kilobases
μM	Micromolar
mM	Millimolar
M	Molar
MD	Minimal defined
MOPS	4-Morpholinepropanesulfonic acid
mRNA	Messenger ribonucleic Acid
nM	Nanomolar
ONPG	2-nitrophenyl β-D-galactopyranoside
ORF	Open reading frame
OD	Optical density
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
SD	Synthetic defined
TAE	Tris acetate electrophoresis buffer
TE	Tris EDTA buffer

Tris-Cl	Tris (hydroxymethyl) aminomethane adjusted to pH with hydrochloric acid
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume
YPD	Yeast Peptone Dextrose (media)

Chapter 1
General introduction

Candida albicans is a commensal organism of humans that can cause opportunistic infections when the host's immune system is compromised. Severe systemic candida infections are difficult to treat successfully and mortality is high, despite the use of anti-fungal therapy. Copper is an essential nutrient for *C. albicans* and is required for important reactions in the cell including respiratory functions and protection from oxidative stress (Hwang *et al.*, 2002; Marvin *et al.*, 2004). Proteins that require copper are also important for the virulence of *C. albicans*, including superoxide dismutase and the ferrous iron transport complex (Hwang *et al.*, 2002; Knight *et al.*, 2002; Ramanan & Wang, 2000). The expression of copper metabolism genes alters in response to host factors such as macrophages and prostaglandin, suggesting that copper metabolism has an important part to play in the response of *C. albicans* to the host environment (Levitin & Whiteway, 2007; Lorenz *et al.*, 2004).

Previous work in our laboratory identified the copper-responsive transcriptional activator CaMac1p, which induces transcription of the high affinity copper transporter gene *CaCTR1* in copper starvation conditions (Marvin *et al.*, 2004). The work described in this thesis identifies further genes regulated by CaMac1p and investigates the promoter elements required for regulation of its target genes. This general introduction describes the role of copper in *C. albicans* and includes a review of copper and iron homeostasis in *C. albicans* and other organisms. Copper and iron metabolism has been most widely studied in the model eukaryote *Saccharomyces cerevisiae* and this organism is therefore referred to extensively throughout the introduction. The introduction concludes with a description of the background to the project and the project's objectives.

1.1 *Candida albicans*

Candida albicans is an obligate commensal fungus found in the gut and mucosal membranes of mammals, but can become pathogenic if the host's immune system is compromised. The fungus is able to grow in the three different morphological forms of yeast, pseudohyphae and hyphae (Sudbery *et al.*, 2004). The process of switching between yeast and filamentous forms of growth is extremely complex and involves a number of regulatory cascades that respond to different environmental signals (Dhillon *et al.*, 2003). While the signals that trigger hyphal switching are not fully understood, filamentous growth can be induced in the laboratory by serum, growth at 37 °C or elevated pH (Sudbery *et al.*, 2004). These growth

conditions bear more similarity to the host environment than laboratory culture conditions, and the inability to grow in both yeast and hyphal forms is associated with decreased virulence (Bendel *et al.*, 2003; Lo *et al.*, 1997; Murad *et al.*, 2001b). However, the transcription factors and environmental conditions that regulate morphogenesis also regulate a number of other genes that are involved in virulence, and it is not clear how exactly the yeast-hyphal switch contributes to the virulence of *C. albicans* (Lane *et al.*, 2001; Murad *et al.*, 2001a).

C. albicans is usually a diploid organism, but possesses two mating-type loci and is able to undergo mating under specific laboratory conditions when these loci are homozygous, reviewed in (Magee & Magee, 2004). The majority of clinical isolates are heterozygous for the mating type loci and heterozygosity appears to confer a competitive advantage that results in increased virulence (Lockhart *et al.*, 2005). This suggests that mating occurs at a low frequency in nature, although recent analysis of the evolutionary history of *C. albicans* has revealed some evidence of sexual reproduction (Odds *et al.*, 2007).

The diploid genome sequence of *C. albicans* was published in 2004 and has proved to be a valuable resource for the *Candida* research community (Jones *et al.*, 2004). The sequence data has been reviewed and updated a number of times and the latest assembly of the genome was published in April 2007 (van het Hoog *et al.*, 2007). Sequence data can be readily accessed and analysed via the Candida Genome Database website (www.candidagenome.org) or the Candida DB website (http://genolist.pasteur.fr/CandidaDB) (Arnaud *et al.*, 2005; Arnaud *et al.*, 2007; d'Enfert *et al.*, 2005). The sequenced *C. albicans* strain, SC5314, was originally isolated from a bloodstream infection and is the parental strain for the majority of laboratory strains used for genetic analysis (Gillum *et al.*, 1984; Jones *et al.*, 2004).

1.2 *C. albicans* and disease

The majority of infections caused by *C. albicans* are minor subcutaneous candidiasis of the oral and vaginal mucosa, commonly known as thrush. The incidence of vaginal candidiasis is very high, and up to 75 % of women will have at least one infection during their lifetime (Ferrer, 2000). *Candida* species can also cause life-threatening systemic disease and they are the fourth most common causative agents of bloodstream infections in the USA (Wisplinghoff *et al.*, 2004). Currently, most candidaemia is caused by *Candida albicans*, and the mortality rate of *Candida* bloodstream infections was recently estimated at 49 % (Gudlaugsson *et al.*, 2003; Pfaller & Diekema, 2002; Tortorano *et al.*, 2004). A contributory factor to high mortality rates is the emergence of antifungal resistance. In contrast to the mechanism of

drug resistance in bacteria, resistance to antifungal drugs emerges within a single *C. albicans* population over time and is not transferred from other populations by mobile genetic elements, reviewed in (Anderson, 2005). The majority of antifungals, including amphotericin B and the widely-used azole class of drugs, target the fungal cell membrane component ergosterol. Resistance to the azole drug fluconazole is especially common due to its widespread use for the treatment of thrush and its fungistatic mechanism of action, which increases the chances that surviving *C. albicans* populations are fluconazole resistant after treatment.

C. albicans infection is associated with risk factors that decrease the competence of the patient's immune system such as antibiotic treatment, surgery, intravascular catheterisation and immunosuppressant drugs (Tortorano *et al.*, 2004). Several properties of *C. albicans* are also associated with the change from commensal organism to pathogen including the ability to switch between yeast and hyphal forms, adherence to surfaces 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 microorganisms 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 reductive high affinity iron uptake system is essential for virulence in the mouse systemic model of candidiasis (Ramanan & Wang, 2000). Copper and iron homeostasis in *C. albicans* are intrinsically linked due to the requirement of copper for high affinity iron uptake (Knight *et al.*, 2002). Previous work in our laboratory has shown that when copper uptake is impaired, high affinity iron uptake is also defective, suggesting that copper accumulation may play a role in the disease process (Marvin *et al.*, 2004). Copper is also required for the superoxide dismutase activity of CaSod1p, and deletion of *CaSOD1* reduces virulence in the systemic mouse model (Hwang *et al.*, 2002).

In addition to roles in iron uptake and superoxide dismutase, a number of recent transcriptional profiling experiments have indicated further roles for copper transport and metabolism genes including 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 ferric reductase-like gene orf19.6139 is upregulated in response to macrophages in both transcriptional profiling and proteomic studies (Fernandez-Arenas *et al.*, 2007; Lorenz *et al.*, 2004). We have previously demonstrated that filamentous and invasive growth of *C. albicans* is increased at low copper levels, and that a *Cactr1*Δ/*ctr1*Δ mutant grows predominantly as hyphae due to the very low

levels of copper taken up by this mutant (Marvin *et al.*, 2003; Marvin *et al.*, 2004). Heterologous expression of *CaMAC1* in *S. cerevisiae* also induces filamentous and invasive growth, suggesting that in copper starvation conditions, *CaMAC1* may activate genes involved in hyphal growth (Huang, 2006). 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.

1.3 Copper and iron

Copper and iron are essential nutrients for almost all living organisms and are important for a wide variety of cellular processes, reviewed in (Crichton & Pierre, 2001). The high redox potential of these transition metals makes them ideal as components of proteins involved in electron transfer such as cytochrome *c* oxidase which requires copper, haem, magnesium and zinc molecules to function (Tsukihara *et al.*, 1995). Conditions of copper or iron starvation therefore cause a shortage of co-factors for many proteins and *S. cerevisiae* mutants lacking iron or copper transporters are therefore unable to grow on respiratory carbon sources (Askwith *et al.*, 1994; Dancis *et al.*, 1994b). In contrast, free copper and iron in the cell can produce toxic hydroxyl radicals that damage DNA and proteins (Urbanski & Beresewicz, 2000). Copper and iron homeostasis must therefore be tightly regulated to maintain these metals at the correct physiological concentrations but without causing any toxic effects to the cell.

A number of strategies for protection against toxic levels of iron and copper have been described in *S. cerevisiae* including storage of excess iron and copper in the vacuole and the intracellular accumulation of ferric-ferrichrome (Moore *et al.*, 2003; Szczypka *et al.*, 1997). Copper chaperones are also used to transport copper through the cytosol without increasing the level of free copper ions (Field *et al.*, 2002). If the concentration of copper does reach potentially toxic levels, it is chelated by the cytosolic copper-binding protein metallothionein (Liu & Thiele, 1997). Any reactive oxygen species that may have been produced are detoxified by the Cu/Zn superoxide dismutase Sod1p (Bermingham-McDonogh *et al.*, 1988). *C. albicans* also prevents copper toxicity by producing metallothioneins and by the detoxification activity of superoxide dismutase (Hwang *et al.*, 2002; Oh *et al.*, 1999; Weissman *et al.*, 2000). An additional strategy used by *C. albicans* for protection from copper toxicity is the transport of metal ions out of the cell using a P-type ATPase extrusion pump. This additional detoxification mechanism may explain why *C. albicans* has a higher tolerance of copper than *S. cerevisiae* (Riggle & Kumamoto, 2000; Weissman *et al.*, 2000).

1.4 Acquisition of iron from the environment

Due to the toxicity of copper and iron, the acquisition of these metals is controlled by highly specific transport systems which facilitate the uptake of enough metals for nutritional purposes, but prevent accumulation of potentially toxic concentrations of metal inside the cell. Several forms of iron are available to fungi including ferrous iron, ferric iron and iron bound to low molecular weight iron-binding molecules known as siderophores (Van Ho *et al.*, 2002). Figure 1.1 summarises the different pathways used by *S. cerevisiae* to acquire iron from these sources. In this section, the iron uptake systems of *S. cerevisiae* will be discussed and compared to our knowledge of iron uptake in *C. albicans* and other fungi.

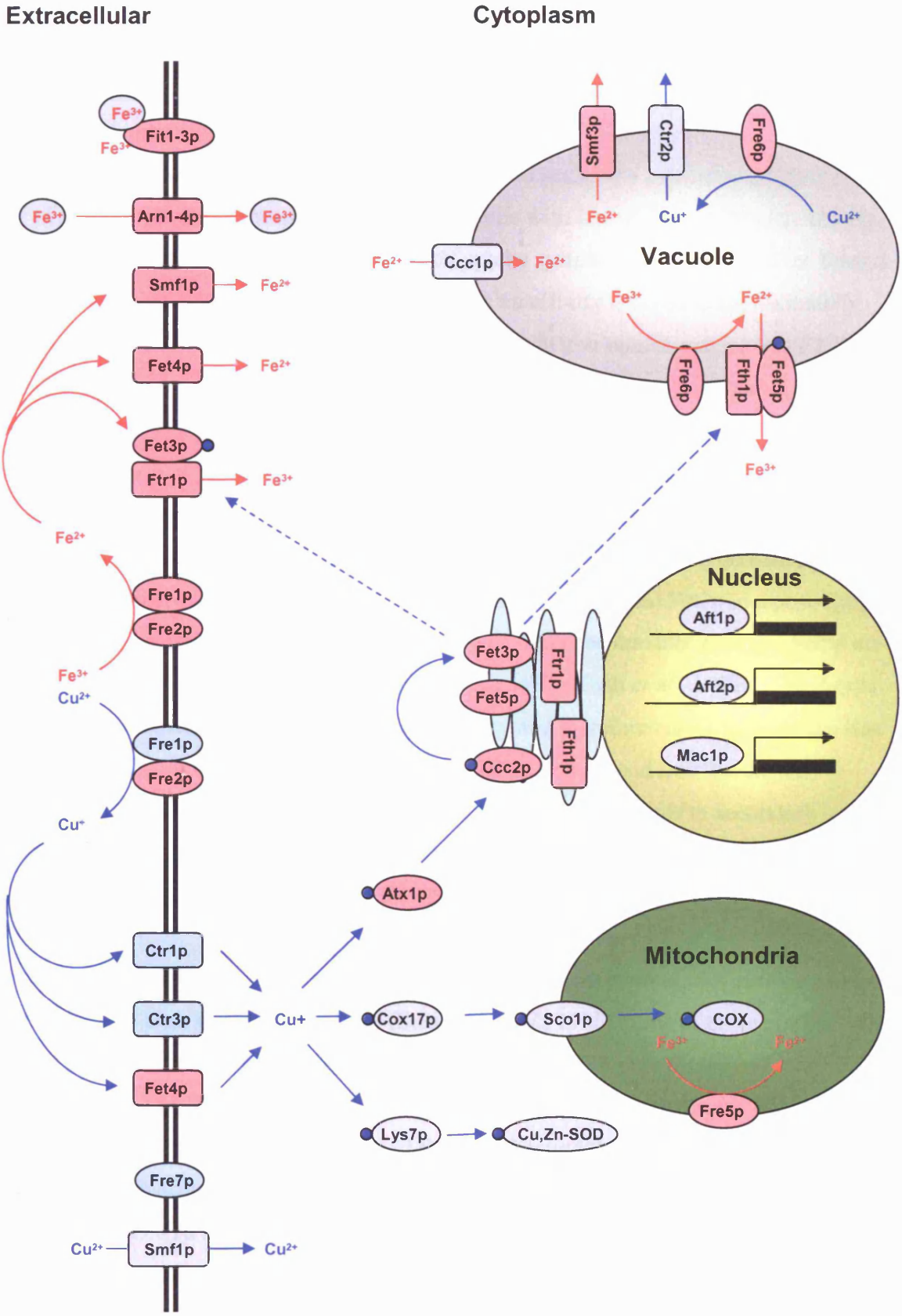
The ferric reductases

Iron is usually available in its insoluble ferric form, and reduction to the more soluble ferrous iron is facilitated in eukaryotes by a number of cytochrome-like proteins termed ferric reductases (Pierre *et al.*, 2002). The *S. cerevisiae* Fre1p and Fre2p reductases are responsible for the majority of cell surface ferric reductase activity, and *fre1Δfre2Δ* mutants are deficient in high affinity iron uptake (Dancis *et al.*, 1990; Georgatsou & Alexandraki, 1994). Transcription of *FRE1* and *FRE2* is increased in low iron conditions, but *FRE1* transcription is also regulated by copper levels, providing a link between the homeostasis of these two essential metals (Dancis *et al.*, 1990; Georgatsou & Alexandraki, 1994; Hassett & Kosman, 1995).

There are a further seven reductases which are less well studied than Fre1/2p but all contain functional domains involved in reduction (Georgatsou & Alexandraki, 1999). Transcription of *FRE3-6* are regulated by iron levels, *FRE7* is regulated by copper levels, and *YGL160w* and *YLR047c* are not regulated by either iron or copper levels (Georgatsou & Alexandraki, 1999; Martins *et al.*, 1998). In contrast to Fre1p and Fre2p, the remaining reductases do not reduce ferric iron at the cell surface but appear to either reduce alternative substrates, or to localise to different parts of the cell. Fre3p and Fre4p reduce iron bound to siderophores at the cell surface, but cannot reduce free ferric iron (Yun *et al.*, 2001). Fre7p is also located on the cell surface and has reductase activity, but its biological function has not yet been determined (Rees & Thiele, 2007; Singh *et al.*, 2007). The function of Fre5p is currently unknown but as it was identified as a member of the mitochondrial proteome it may therefore be involved in iron transport across the mitochondrial membrane (Sickmann *et al.*, 2003). Fre6p is located on the vacuolar membrane and is required for the export of copper from the vacuole by Ctr2p (Rees & Thiele, 2007; Singh *et al.*, 2007). Fre6p may also be the ferric reductase that reduces Fe^{3+} for transport out of the vacuole by the Fet5p/Fth1p high affinity

Figure 1.1 Copper and iron uptake in *S. cerevisiae*

The uptake of copper and iron is intricately linked in the model organism *S. cerevisiae* and several proteins are jointly involved in the metabolism of both metals. Copper and iron are both reduced at the cell surface by the ferric/cupric reductases Fre1p and Fre2p. Fre7p is a plasma membrane reductase that may also be involved in the reduction of copper and iron. Cuprous ions are transported across the plasma membrane by the redundant high affinity copper transporters Ctr1p and Ctr3p. Cuprous ions can also be transported by the low affinity transporter Fet4p, which is also involved in the uptake of Fe^{2+}, Zn^{2+} and Cd^{2+}. An alternative pathway for low affinity copper uptake is the divalent metal ion transporter Smf1p that transports Cu^{2+}, Fe^{2+} and Mn^{2+}. Once inside the cell, copper is transported by copper chaperone proteins including Cox17p and Lys17p, which transport copper through the cytoplasm to be incorporated into cytochrome *c* oxidase (COX) and superoxide dismutase (SOD) respectively. Sco1p facilitates the movement of copper across the mitochondrial membrane and may be involved in direct loading of copper onto cytochrome *c* oxidase. Another copper chaperone, Atx1p, transfers copper to the ATPase Ccc2p which is located in the Golgi membrane. Ccc2p loads copper onto the multicopper oxidases Fet3p and Fet5p. Fet3p forms a complex with Ftr1p which is transferred to the plasma membrane, and this complex is a high affinity ferrous iron transporter. Fet5p and Fth1p form a similar ferrous iron transport complex that localises to the vacuolar membrane. This demonstrates an intrinsic link between copper and iron uptake, because copper is absolutely required for high affinity iron transport. The vacuole is thought to be an intracellular store for both copper and iron. Iron is exported from the vacuole by the high affinity Fet5p/Fth1p complex and the Nramp transporter Smf3p. Iron is transported into the vacuole by Ccc1p but a copper import system has yet to be identified. Copper is exported from the vacuole by the high affinity transporter Ctr2p which requires the vacuolar membrane cupric reductase Fre6p, indicating that Ctr2p transports Cu^{+} ions. Fre6p may also reduce iron for export by Fet5p/Fth1p but this has not yet been directly proven. An additional ferric reductase, Fre5p is located in the mitochondrion. The transcriptional activator Mac1p induces transcription in low copper conditions and the proteins encoded by these genes are shown in blue. The transcriptional activators Aft1p and Aft2p induce transcription in low iron conditions and the proteins encoded by these genes are shown in red. Note that the *FRE1* gene is activated by Mac1p and Aft1p/Aft2p, providing an additional link between copper and iron uptake. Figure adapted from Mason, 2006.


Extracellular
Cytoplasm
Fe3+
Fit1-3p
Fe3+
Fe3+
Arn1-4p
Fe3+
Smf1p
Fe2+
Fet4p
Fe2+
Fet3p
Ftr1p
Fe3+
Fe2+
Fre1p
Fre2p
Fe3+
Cu2+
Fre1p
Fre2p
Cu+
Ctr1p
Ctr3p
Fet4p
Fre7p
Cu2+
Smf1p
Cu2+
Smf3p
Ctr2p
Fre6p
Fe2+
Cu+
Cu2+
Fe2+
Ccc1p
Fe2+
Vacuole
Fe3+
Fe2+
Fre6p
Fth1p
Fet5p
Fe3+
Nucleus
Aft1p
Aft2p
Mac1p
Fet3p
Ftr1p
Fet5p
Fth1p
Ccc2p
Atx1p
Mitochondria
Ctr1p
Ctr3p
Fet4p
Cu+
Cox17p
Sco1p
COX
Fe3+
Fe2+
Fre5p
Lys7p
Cu,Zn-SOD

iron transport complex (Urbanowski & Piper, 1999). The cellular location and function of *YGL160w* and *YLR047c* are as yet unknown, but a mutant strain lacking *YLR047c* demonstrates slow growth in low iron conditions, indicating a potential role in copper or iron homeostasis for this gene (De Freitas *et al.*, 2004).

High affinity iron uptake in *S. cerevisiae*

After reduction at the cell surface, iron can be transported across the plasma membrane by either of two independent ferrous transport systems, one with low and one with high affinity for iron (Eide *et al.*, 1992). The high affinity iron uptake system is highly specific for ferrous iron and is only active in low iron conditions as it has an affinity for iron of approximately 0.15 μM (Eide *et al.*, 1992). The first gene identified in the iron uptake system was *FET3* (Ferrous Transport 3), and *fet3*Δ mutants are defective for high affinity iron uptake, grow slowly in low iron and low copper media and cannot grow using non-fermentable carbon sources (Askwith *et al.*, 1994). However, Fet3p is not an iron transporter but is a member of the multicopper oxidase protein family (Askwith *et al.*, 1994). The oxidase activity of Fet3p is essential for iron uptake, and ferrous iron is the best substrate for Fet3p (Askwith & Kaplan, 1998; de Silva *et al.*, 1997). Four copper ions are loaded onto Fet3p in a post-Golgi compartment and copper is essential for the function of Fet3p, as mutants with apo-Fet3p are deficient for high affinity iron uptake (Stearman *et al.*, 1996; Yuan *et al.*, 1997). The P-type ATPase Ccc2p is essential for copper-loading of Fet3p, and therefore *ccc2*Δ mutants are also defective in high affinity iron uptake (Yuan *et al.*, 1995). Copper and iron uptake are therefore intrinsically linked in *S. cerevisiae*, and copper starvation leads to secondary iron starvation because copper is absolutely required for iron uptake (Askwith *et al.*, 1994; Dancis *et al.*, 1994b).

Additional experiments identified two proteins on the plasma membrane of *S. cerevisiae* that were required for high affinity iron uptake (Stearman *et al.*, 1996). One of these proteins was Fet3p and the other protein was a permease encoded by the *FTR1* gene (Stearman *et al.*, 1996). Expression of *FET3* was required for localisation of Ftr1p at the plasma membrane and *FTR1* was required for copper-loading of Fet3p, indicating that both proteins must be functional for high affinity iron uptake to take place (Stearman *et al.*, 1996). Conserved iron binding motifs in Ftr1p are required for iron uptake, suggesting that Ftr1p is able to directly bind iron (Stearman *et al.*, 1996). A similar complex of a multicopper oxidase, Fet5p, and an iron permease, Fth1p, is found at the vacuolar membrane and transports iron out of the vacuole (Spizzo *et al.*, 1997; Urbanowski & Piper, 1999).

Low affinity iron uptake in *S. cerevisiae*

Mutants that have deficiencies in high affinity iron uptake are still able to grow normally when supplemented with iron because of the activity of the low affinity plasma membrane iron transporter Fet4p, which functions independently of the Fet3p/Ftr1p system (Dix *et al.*, 1997; Dix *et al.*, 1994). The low affinity iron uptake system has an affinity of 30 μM, compared to an affinity of 0.15 μM for the high affinity system, facilitating growth in iron-rich conditions where the high affinity system is not functional (Dix *et al.*, 1994; Eide *et al.*, 1992). Low affinity iron uptake is also induced in anaerobic conditions when the high affinity system is not functional because oxygen is required for the oxidase activity of Fet3p (de Silva *et al.*, 1997; Hassett *et al.*, 1998; Jensen & Culotta, 2002). Iron transport by Fet4p requires reduction of ferric iron by ferric reductases or ascorbate and Fet4p is also able to transport other transition metals including Mn^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+} and Cu^{+} (Dix *et al.*, 1997; Dix *et al.*, 1994). This evidence suggests that iron is transported by Fet4p as Fe^{2+}.

Smf1p is a general metal ion transporter located on the plasma membrane, and overexpression of *SMF1* can restore ferrous iron uptake to a *fet3Δfet4Δ* mutant (Cohen *et al.*, 2000). Smf1p is a member of the Nramp family of metal transporters, and the homologous protein Smf3p is involved in low affinity iron transport out of the vacuole, independent of the high affinity Fth1p/Fet5p complex (Portnoy *et al.*, 2000). Transport of iron into the vacuole is also mediated by a low affinity transporter, Ccc1p (Li *et al.*, 2001). The yeast vacuole is important for the maintenance of copper and iron homeostasis and the prevention of metal toxicity (Szczypka *et al.*, 1997). It is thought that iron is stored in the vacuole during iron replete conditions to prevent toxicity, and this reserve of iron is exported from the vacuole into the cytoplasm during iron starvation conditions (Li *et al.*, 2001; Portnoy *et al.*, 2000; Raguzzi *et al.*, 1988; Urbanowski & Piper, 1999). Iron is also stored in the cytoplasm bound to the siderophore ferrichrome, and this form of iron is available for use in cellular iron metabolism (Moore *et al.*, 2003). Ferrichrome is also used as both an iron source and iron strorage protein in *Schizosaccharomyces pombe* (Schrettl *et al.*, 2004).

Siderophore iron transport in *S. cerevisiae*

S. cerevisiae does not produce siderophores but is able to utilise siderophores from a variety of bacteria and fungi as the sole iron source (Lesuisse *et al.*, 1998). Siderophores can be transported via reductive or non-reductive pathways, but only rhodotorulic acid is solely taken up by the reductive pathway (Lesuisse & Labbe, 1989; Yun *et al.*, 2000b). In the reductive uptake pathway, the ferric iron bound to siderophores is reduced at the plasma membrane by the ferric reductases Fre1-4p. The ferrous iron released from the siderophore is then

transported into the cell by the ferrous iron transporter complex (Lesuisse *et al.*, 1987; Stearman *et al.*, 1996; Yun *et al.*, 2001). Fre1p and Fre2p can reduce iron from a wide range of siderophores including ferrichrome, ferrioxamine B, rhodotorulic acid and fusarines (Yun *et al.*, 2001). However, Fre3p can reduce ferrichrome and rhodotorulic acid and Fre4p can only reduce rhodotorulic acid (Yun *et al.*, 2001). The specificity of the reducase substrates reflects their affinities for iron, i.e. Fre4p has the lowest affinity and Fre1p the highest affinity for iron (Yun *et al.*, 2001). The reductive system is more active at higher concentrations of siderophores where the non-reductive system, which involves specific transporter proteins with very high affinity, can easily become saturated with siderophores (Lesuisse & Labbe, 1989; Moore *et al.*, 2003; Yun *et al.*, 2000a; Yun *et al.*, 2000b).

Four transporter proteins that can transport ferric-siderophores have been identified and each of these has different specificities for siderophore uptake (Yun *et al.*, 2000a). Arn1p appears to have the least specificity and can transport ferrichrome, ferrichrome A, ferrirubin and ferrirhodin (Heymann *et al.*, 2000a; Yun *et al.*, 2000b). Iron-loaded siderophores are transported by Arn1p across the plasma membrane and remain stable in the cytoplasm (Moore *et al.*, 2003). Arn2p is specific for triacetylfusarinine C, whereas the substrates for Arn3p are ferrioxamine B, ferrichrome and ferrichrome A which overlap with those of Arn1p and other transporters (Heymann *et al.*, 1999; Lesuisse *et al.*, 1998; Yun *et al.*, 2000a; Yun *et al.*, 2000b). Arn4p is required for transport and utilisation of the bacterial siderophore enterobactin, confirming that *S. cerevisiae* is able to use both hydroxamate and catecholate siderophores as iron sources (Heymann *et al.*, 2000b). The transport of ferric iron and siderophores is also enhanced by the presence of the cell wall mannoproteins Fit1-3p (Protchenko *et al.*, 2001). The Fit proteins are thought to bind iron and siderophores in the cell wall, which may enhance iron uptake by increasing the concentration of iron available for transport across the plasma membrane or may facilitate transport of the relatively large siderophore molecules across the cell wall (Protchenko *et al.*, 2001).

High affinity iron uptake in *C. albicans*

The *C. albicans* genome contains a number of multi-gene families, including the secreted aspartyl proteases, ABC transporters, ferric reductases, multicopper oxidases and iron permeases (Braun *et al.*, 2005). Different members of the multi-gene families are expressed in different conditions, such as during hyphal growth or at different pH values, and this may be an adaptation to the variety of niches that *C. albicans* can occupy as a commensal organism. The members of the iron uptake gene families may also be expressed in different iron concentrations, in response to different iron sources or in various cellular locations.

The *C. albicans* iron permease genes *CaFTR1* and *CaFTR2* show high levels of homology to *ScFTR1* and are able to rescue *S. cerevisiae ftr1*Δ mutants, suggesting that the high affinity iron uptake systems are similar in these two organisms (Ramanan & Wang, 2000; Stearman *et al.*, 1996). Although CaFtr2p appears to function as an iron transporter when heterologously expressed in *S. cerevisiae*, a *C. albicans ftr2*Δ/ *ftr2*Δ mutant shows wild-type levels of iron uptake (Ramanan & Wang, 2000). CaFtr1p localises to the plasma membrane and the *ftr1*Δ/ *ftr1*Δ mutant has almost no iron uptake activity and cannot grow in low iron conditions (Ramanan & Wang, 2000). The transcription of *CaFTR1* is increased in low iron conditions and transcription of *CaFTR2* is induced in high iron conditions (Ramanan & Wang, 2000). This evidence suggests that CaFtr1p, and not CaFtr2p, is the major iron permease in *C. albicans* and is the functional homologue of ScFtr1p. The *ftr1*Δ/ *ftr1*Δ mutant cannot establish a systemic infection in a mouse model of candidiasis, and this clearly shows the importance of effective iron uptake in the growth and virulence of *C. albicans* (Ramanan & Wang, 2000).

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 in *S. cerevisiae* (Knight *et al.*, 2002). The copper transporter gene *CaCTR1* is also required for effective iron uptake, confirming the need for adequate copper levels to incorporate into multicopper oxidases (Marvin *et al.*, 2004). However, copper is not required for iron uptake from siderophores, suggesting the presence of an alternative mechanism of iron uptake (Knight *et al.*, 2002). There are five homologues of the *FET3* gene including *CaFET3,* which is able to complement a *S. cerevisiae fet3*Δ mutant and which is also required for growth of *C. albicans* in low iron media (Braun *et al.*, 2005; Eck *et al.*, 1999). Although CaFet3p is the most likely candidate for the formation of a permease/oxidase complex in *C. albicans*, the effect of a *CaFET3* mutation on high affinity iron uptake has not been determined and deletion of *CaFET3* has no effect on virulence in the mouse model of candidiasis (Eck *et al.*, 1999). One or more of the other multicopper oxidases in *C. albicans* may therefore be involved in iron uptake. *CaFET99* is the only other multicopper oxidase gene to be investigated so far and mutation of this gene has no effect on iron uptake (Knight *et al.*, 2002).

Ferric reductases in *C. albicans*

Work by our laboratory and others has demonstrated that the ferric reductase gene *CaFRE10* (orf19.1415) is the major cell surface ferric and cupric reductase in *C. albicans* and is required

for wild type levels of high affinity iron uptake (Knight *et al.*, 2002; Mason, 2006). Further work in our laboratory has indicated that CaFre2p (orf19.1264) has a low level of cell surface ferric reductase activity in iron limiting conditions and may play a similar role to ScFre2p (Mason, 2006). The *FRE1* gene (orf19.1263) is able to complement a *S. cerevisiae* *fre1Δfre2Δ* mutant, but a *C. albicans fre1Δ/fre1Δ* mutant does not show any obvious phenotypes compared with the wild type (Hammacott *et al.*, 2000; Mason, 2006). There are a further 14 ferric reductase-like genes in the *C. albicans* genome, but as yet no additional roles have been determined for their protein products (Braun *et al.*, 2005). Transcriptional profiling experiments have shown that transcription of a number of ferric reductase genes is altered in response to iron limitation, prostaglandin or engulfment by macrophages, suggesting roles for the ferric reductases in iron transport and responses to host factors (Lan *et al.*, 2004; Levitin & Whiteway, 2007; Lorenz *et al.*, 2004).

Alternative sources of iron for *C. albicans*

C. albicans mutants defective in high affinity iron uptake are able to grow in high iron concentrations, indicating the presence of a low affinity iron transporter and/or utilisation of intracellular iron stores , however as yet no evidence has been found for these processes (Ramanan & Wang, 2000). There is no homologue of the *S. cerevisiae* low affinity transporter gene *FET4* in *C. albicans* but it is possible that the CaFtr2p transporter may function as a low affinity transporter (Ramanan & Wang, 2000).

C. albicans is able to use iron from sources such as siderophores, transferrin, haemoglobin and haemin (Brock *et al.*, 1991; Ismail & Lupan, 1986; Manns *et al.*, 1994). Several reports have described the secretion of siderophores of the hydroxamate and phenolate types, but no siderophore biosynthesis genes have been identified in the *C. albicans* genome (Haas, 2003; Hannula *et al.*, 2000; Holzberg & Artis, 1983; Ismail *et al.*, 1985; Sweet & Douglas, 1991). This discrepancy may be due to strain variation, as 20 % of *C. albicans* strains tested in one study did not produce siderophores (Hannula *et al.*, 2000). Another important consideration is the source of isolates because the sequenced strain SC5314 was isolated from a bloodstream infection (Gillum *et al.*, 1984), whereas clinical isolates that produced siderophores were obtained from superficial mucosal sites (Hannula *et al.*, 2000). Siderophore production may therefore be an adaptation to the environmental niche occupied by the organism.

Despite our lack of knowledge about siderophore production in *C. albicans* the mechanisms of siderophore uptake are well-characterised. *C. albicans* is able to use ferrioxamine B and E,

triacetylfusarinine C or ferrichrome-type siderophores as a sole iron source (Lesuisse *et al.*, 1998). Multiple transporters are used in siderophore uptake in most fungi, but only one siderophore transporter has been identified in *C. albicans* (Haas, 2003). This is unusual when compared to the expansion of many gene families in *C. albicans*, and may reflect a more limited range of siderophores that are encountered by *C. albicans*. It is also possible that the principal function of this single transporter is to transport the siderophores produced by C. albicans, but is also able to facilitate iron acquisition from siderophores produced by other species.

Deletion of the *ScARN1* homologue *CaARN1* reduces the uptake of iron from ferrichrome-type siderophores, and the reductive iron uptake system is required for the remaining uptake of ferrichrome (Heymann *et al.*, 2002; Hu *et al.*, 2002). Uptake of ferrioxamine B is dependent on CaFtr1p and CaFtr2p, but is independent of CaArn1p, indicating that iron is removed from this siderophore extracellularly prior to uptake by the reductive high affinity iron uptake system (Heymann *et al.*, 2002; Hu *et al.*, 2002). Homozygous deletion mutants of *CaARN1* did not show any difference from wild type in the mouse model of systemic infection, but *arn1Δ/ arn1Δ* mutants showed decreased virulence in a model of oral mucosal infection (Heymann *et al.*, 2002; Hu *et al.*, 2002). The evidence available on the production and uptake of siderophores in *C. albicans* suggests that siderophores may play a more important role in mucosal candidiasis than in systemic candidiasis.

Transferrin, haemin and haemoglobin can all be used as sole iron sources by *C. albicans*, and may be important iron sources during systemic infection and dissemination via the bloodstream (Brock *et al.*, 1991; Manns *et al.*, 1994). This is relevant to the mouse model of systemic candidiasis where mice are injected with *C. albicans* directly into the tail vein (de Repentigny, 2004). Transferrin is reduced at the cell surface and the free ferrous iron is taken up by the reductive iron uptake system (Knight *et al.*, 2005). The *CaFRE10* ferric reductase gene is required for the reduction of transferrin in medium of pH 4.3, but *CaFRE10* is not required at the higher pH of 6.3 (Knight *et al.*, 2005). Alternative ferric reductases are therefore likely to be active in the reduction of transferrin at physiological pH and may be important during systemic candidiasis.

1.5 Regulation of iron uptake

Iron homeostasis must be tightly regulated to prevent toxicity from elevated iron levels, but enough iron must still be available for essential cellular processes such as respiration. The majority of iron homeostasis genes in *S. cerevisiae* are under the control of the paralogous

transcription factors Aft1p and Aft2p (Rutherford *et al.*, 2001; Rutherford *et al.*, 2003; Yamaguchi-Iwai *et al.*, 1995). Aft1p and Aft2p are transcriptional activators that increase the initiation of transcription by RNA polymerase II and the general transcription factors (Fragiadakis *et al.*, 2004). Transcriptional repressors can also negatively regulate transcription by repressing the initiation of this process. The first step in the initiation of transcription is the binding of TATA-box binding protein (TBP) and TBP- associated factors (TAFs) which together make up the general transcription factor TFIID. This is followed by recruitment of the other general transcription factors (IIB, IIE, IIF and IIH) and RNA polymerase II, reviewed in (Roeder, 1996). The Mediator complex directly interacts with the C-terminal domain of RNA polymerase II to increase the efficiency of basal transcription and Mediator is also likely to be the intermediate between specific transcription factors, such as Aft1p, and the transcriptional machinery (Bjorklund & Gustafsson, 2005). It is thought that transcriptional activators recruit Mediator and the general transcription factors to increase the initiation of transcription, as illustrated in Figure 1.2 (i). Only Mediator without the Srb (suppressor of RNA polymerase B) sub-units 8-11 can initiate transcription, and repressors such as Tup1p may therefore specifically recruit Mediator containing Srb8-11 to negatively regulate transcription as illustrated in Figure 1.2 (ii).

The specificity of transcriptional activators and repressors is determined by the DNA binding domain of the protein which locates the transcription factor in the region of the relevant promoter. The high degree of sequence specificity is reflected in the wide variety of DNA binding domains including zinc finger, helix-turn-helix and leucine zipper structures (Latchman, 1991). Regulation of transcription in response to altered stimuli such as iron concentration or temperature is due to alterations in the activity of the transcription factors. This can be due to conformational change from an inactive to an active form or a result of regulation at a number of levels including transcriptional regulation, post-translational modification of the protein or degradation of functional transcription factors. The iron-responsive activity of Aft1p is due to the localisation of Aft1p which is located in the nucleus and can activate its target genes only in low iron conditions (Yamaguchi-Iwai *et al.*, 2002). In high iron conditions, Aft1p interacts with the nuclear export receptor Msn5p and this iron-dependent interaction facilitates rapid export of Aft1p into the cytoplasm which prevents gene activation by Aft1p (Ueta *et al.*, 2007).

Aft1p and Aft2p have overlapping but distinct roles in iron regulation, and also regulate a significant number of genes that are not involved in iron regulation or have unknown functions (Rutherford *et al.*, 2003). Common genes regulated by both transcription factors

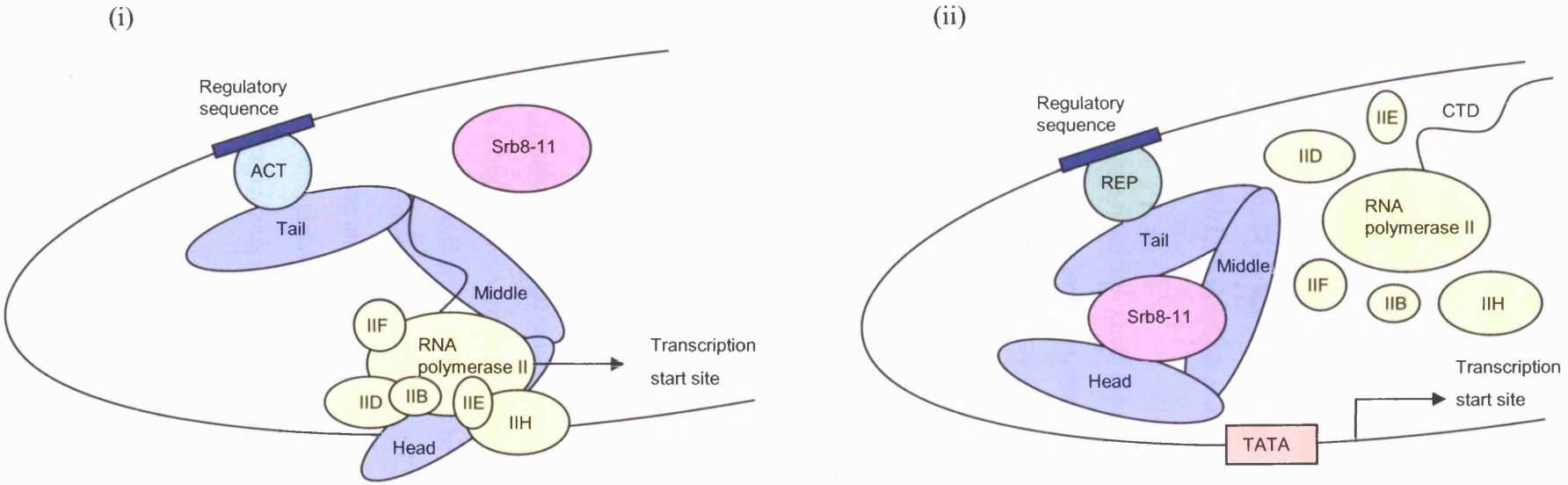


Figure 1.2 Models of activation (i) and repression (ii) of the transcriptional apparatus in yeast.
Sequence-specific activators (ACT, blue) or repressors (REP, green) bind regulatory sequences upstream of the TATA box. Activators and repressors interact with sub-units of the tail domain of the Mediator complex (purple). Activators recruit Mediator that is not associated with the Srb8-11 complex (pink) and this form of Mediator can associate with the C-terminal domain (CTD) of RNA polymerase II to initiate transcription via the general transcriptional machinery (yellow). Repressors such as Tup1 recruit Mediator containing the Srb8-11 complex and this prevents association with RNA polymerase II and the initiation of transcription by the general transcriptional machinery (yellow). Adapted from Bjorklund & Gustafsson (2005).

include *FIT1-3, FRE1, FET3* and *FTR1* which are all required for high affinity iron uptake (Rutherford *et al.*, 2003). Additional iron uptake genes that are only regulated by Aft1p include the siderophore transporter genes *ARN1-4,* the low affinity iron transporter *FET4* and the ferric reductase genes *FRE2-6* (Fragiadakis *et al.*, 2004; Jensen & Culotta, 2002; Martins *et al.*, 1998; Yun *et al.*, 2000a). The copper chaperone gene *ATX1* and the ATP-ase gene *CCC2* are also upregulated by Aft1p to ensure a constant supply of copper to the iron permease/oxidase complex (Lin *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1996). Genes solely regulated by Aft2p include *SMF3, FET5*, the mitochondrial iron transporter *MRS4* and the iron-sulphur assembly gene *ISU1*, and these genes are all involved in intracellular iron transport (Portnoy *et al.*, 2002; Rutherford *et al.*, 2001; Rutherford *et al.*, 2003).

Deletion of the *AFT1* gene results in phenotypes associated with defects in high affinity iron uptake including slow growth in low iron, respiratory deficiency, sensitivity to oxidative stress, low levels of iron transport and low ferric reductase activity (Casas *et al.*, 1997; Yamaguchi-Iwai *et al.*, 1995). These phenotypes are a consequence of lower expression of the Aft1p target genes including *FTR1, FET3* and *FRE1*. An *aft2*Δ mutant does not show any difference from wild type when the same phenotypes are tested, but an *aft1*Δ*aft2*Δ mutant is more sensitive to iron starvation and oxidative stress than an *aft1*Δ mutant, suggesting that *AFT2* does have some role in iron homeostasis (Blaiseau *et al.*, 2001). Overexpression of *AFT2* can rescue *aft1*Δ mutants, and results in increased expression of *FET3* and *FTR1*, indicating that Aft2p can partially compensate for a lack of Aft1p and that the roles of the two transcription factors are partially redundant (Blaiseau *et al.*, 2001; Courel *et al.*, 2005; Rutherford *et al.*, 2001). The two proteins bind the same core sequence of GCACCC in the promoters of target genes in low iron conditions (Courel *et al.*, 2005; Rutherford *et al.*, 2001). However, Aft1p has a higher DNA binding affinity than Aft2p and is a stronger activator (Courel *et al.*, 2005; Rutherford *et al.*, 2001). The binding of Aft1p is also more specific and requires the slightly extended sequence TGCACCCA (Courel *et al.*, 2005). The exact mechanism of DNA binding and gene activation by Aft1p is currently unknown, but Aft1p requires the transcriptional architecture protein Nhp6p and co-activator Ssn6p during the activation of *FRE2* (Fragiadakis *et al.*, 2004).

Iron-responsive transcriptional regulation in *C. albicans*

Previous work in our laboratory identified and characterised a *C. albicans* homologue of *AFT1* to determine if the high affinity iron uptake systems were regulated in a similar manner in these two organisms (Mason, 2006). The *Caaft1*Δ/*aft1*Δ mutant did not show any phenotypes associated with defects in high affinity iron uptake and grew as well as the wild

type in low iron media and on respiratory carbon sources (Mason, 2006). The *Caaft1Δ/aft1Δ* mutant also displayed wild-type levels of iron uptake and ferric/cupric reductase activity (Casas *et al.*, 1997; Mason, 2006; Yamaguchi-Iwai *et al.*, 1995). Deletion of *CaAFT1* did not reduce transcription of the ferric reductase genes as would be expected of a functional *ScAFT1* homologue, but transcription of *CaFRE5* and *CaFRE10* was slightly elevated in the *Caaft1Δ/Caaft1Δ* mutant (Mason, 2006). The evidence suggests that CaAft1p does not play a similar role to ScAft1p in the regulation of iron uptake in *C. albicans*, but CaAft1p may still be involved in iron homeostasis in a different capacity.

A *C. albicans* gene that rescues the *S. cerevisiae aft1Δ* mutation was identified as the putative transcription factor gene *IRO1* (Garcia *et al.*, 2001). Expression of *IRO1* in *aft1Δ* allowed the mutant to grow in low iron conditions, however the effect of *IRO1* on additional *aft1Δ* phenotypes or on the regulation of Aft1p target genes was not investigated further (Garcia *et al.*, 2001). In addition, no iron-related phenotypes have been reported for a homozygous null *Cairo1Δ/iro1Δ* mutant (Chibana *et al.*, 2005). The expression of *IRO1* in *S. cerevisiae* may therefore simply suppress the *aft1Δ* mutation rather than functionally complement it, and a role for *IRO1* in *C. albicans* iron homeostasis remains in doubt.

The GATA-type transcriptional repressors

In contrast with activation by Aft1p observed in *S. cerevisae*, iron uptake genes in many fungi are instead repressed in high iron conditions by GATA-type repressors including Urbs1 from *Ustilago maydis*, SRE from *Neurospora crassa,* SREA from *Aspergillus nidulans* and Fep1 from *Schizosaccharomyces pombe* (Oberegger *et al.*, 2001; Pelletier *et al.*, 2002; Voisard *et al.*, 1993; Zhou *et al.*, 1998). In the absence of a functional homologue of Aft1p, the most likely candidate for the regulation of iron homeostasis in *C. albicans* is the GATA-type transcriptional repressor Sfu1p (Lan *et al.*, 2004). The *SFU1* gene was identified as a homologue of *URBS1* and the predicted protein contains features typical of GATA-type transcription factors, including two N-terminal zinc fingers either side of a cysteine-rich region containing four highly conserved cysteine residues (Lan *et al.*, 2004). A microarray study to compare the transcriptional profiles of an *sfu1Δ/ sfu1Δ* mutant with wild type *C. albicans* indicated that Sfu1p represses a wide range of iron homeostasis genes including *CaFRE1-5, CaFRE10, CaARN1* and *CaFTH1* (Lan *et al.*, 2004). Additional functional analysis of the *sfu1Δ/ sfu1Δ* mutant carried out in our laboratory has demonstrated that increased transcription of ferric reductase genes, including *CaFRE5* and *CaFRE10*, does indeed result in an increased level of cell surface ferric and cupric reductase activity (Rose

Wood, personal communication). We have also shown that high affinity iron uptake is elevated in the *sfu1Δ/ sfu1Δ* mutant and that this correlates with increased transcription of the iron permease gene *CaFTR1* (Rose Wood, personal communication).

Recent work has shown that heterologously expressed *SFU1* can rescue the iron-related phenotypes of a *S. pombe fep1Δ* mutant, including repressing the multicopper oxidase gene *fio1*$^+$ and the siderophore transporter gene *str1*$^+$ in high iron concentrations (Pelletier *et al.*, 2007). The N-terminal zinc finger domain of Sfu1p is also able to bind to the same GATA-type sequence as Fep1 in the promoter of *str1*$^+$ that is occupied during *str1*$^+$ repression. Lan *et al.* have suggested that Sfu1p may recruit the global repressor CaTup1p in high iron conditions to act as a co-repressor. A number of the genes regulated by Sfu1p, including *CaARN1*, *CaFRE10* and *CaFTR1,* are also repressed by CaTup1p in high iron conditions (Knight *et al.*, 2002; Lesuisse *et al.*, 2002). Fep1 requires two similar co-repressors, Tup11 and Tup12, to repress its target genes and therefore the hypothesis that CaTup1p acts a co-repressor with Sfu1p is supported by the strong similarity between Sfu1p and Fep1 (Pelletier *et al.*, 2002; Znaidi *et al.*, 2004). Yeast two hybrid studies have demonstrated that Sfu1p interacts with both Tup11 and Tup12, and that *SFU1* is unable to rescue a *fep1Δ/tup11Δ/tup12Δ* mutant (Pelletier *et al.*, 2007). The evidence to date therefore suggests that Sfu1p has transcriptional repressor activity and that it regulates iron homeostasis in *C. albicans* in a similar way to Fep1 in *S. pombe.*

1.6 Copper uptake systems in *C. albicans* and other fungi

Copper uptake is intricately linked with iron uptake and the mechanisms used for acquiring the two metals are similar. Like iron, copper can be transported by high and low affinity uptake systems with copper affinities of 2 μM and 22 μM respectively (Hassett & Kosman, 1995; Hassett *et al.*, 2000). The proteins involved in copper uptake and metabolism in *S. cerevisiae* are illustrated in Figure 1.1 and described in this section of the introduction. The genes and proteins involved in copper uptake are highly conserved, and the *S. cerevisiae* system is compared to other fungi, including *C. albicans,* and higher eukaryotes.

The cupric reductases

Environmental copper is usually found as Cu^{2+} and cell surface cupric reductase activity is required for high affinity copper uptake in *S. cerevisiae*, suggesting that Cu^+ is the substrate for the high affinity copper transporter (Dancis *et al.*, 1994b; Hassett & Kosman, 1995). The cupric reductases Fre1p and Fre2p are responsible for approximately 85-90 % of cell surface cupric reductase activity, with the majority of the activity provided by Fre1p (Georgatsou *et*

al., 1997). Recent investigations have suggested that the reductase encoded by the copper-regulated *FRE7* gene is located on the plasma membrane, and therefore Fre7p may contribute the remaining cupric reductase activity on the cell surface of *S. cerevisiae* (Rees & Thiele, 2007; Singh *et al.*, 2007).

Previous work in our laboratory has described copper and iron-regulated cupric reductase activity in *C. albicans* (Morrissey *et al.*, 1996), and we have also shown that cupric reductase activity is reduced by half in a *fre10Δ/fre10Δ* mutant (Mason, 2006). Cupric reductase activity remains copper-regulated in this mutant but iron regulation of the activity is abolished (Mason, 2006). This suggests that at least one additional copper-regulated cupric reductase is active on the cell surface of *C. albicans*.

High affinity copper uptake in *S. cerevisiae*

The first copper transporter gene to be identified was *S. cerevisiae CTR1*, and *ctr1Δ* mutants demonstrate decreased uptake of radiolabelled copper, slow growth in low copper media, respiratory defects and sensitivity to oxidative stress (Dancis *et al.*, 1994a; Dancis *et al.*, 1994b). The *CTR1* gene was initially identified during a screen for mutants with deficient iron transport activity, and *ctr1* mutants show decreased high affinity iron uptake and slow growth in low copper, emphasising the importance of copper for effective iron uptake (Dancis *et al.*, 1994a; Dancis *et al.*, 1994b). Ctr1p forms homotrimers on the plasma membrane that specifically transport copper (Dancis *et al.*, 1994b; Sinani *et al.*, 2007). An additional high affinity copper transporter gene was also identified (*CTR3*), but *CTR3* is not expressed in most laboratory strains of *S. cerevisiae*, including the strain used to identify *CTR1* (Knight *et al.*, 1996). This is because of a transposon insertion in the promoter of *CTR3*, which prevents transcription of the gene (Knight *et al.*, 1996). The double deletion mutant *ctr1Δ/ctr3Δ* displayed the same phenotypes as the original *ctr1Δ* mutant that was identified by Dancis *et al.*, showing respiratory defects, slow growth in low copper or iron media and no uptake of copper or iron (Knight *et al.*, 1996). In *S. cerevisiae* strains with functional copies of both *CTR1* and *CTR3*, the presence of either gene was sufficient for high affinity copper uptake, although both genes are required for maximal copper uptake activity (Knight *et al.*, 1996).

The copper transporter protein family

Additional copper transporter genes from a wide variety of species were subsequently identified due to their similarity to *CTR1* and *CTR3* including copper transporter genes from *S. pombe*, plants, insects, mice and humans (Kampfenkel *et al.*, 1995; Labbe *et al.*, 1999; Lee *et al.*, 2000; Zhou & Gitschier, 1997; Zhou *et al.*, 2003). Work in our laboratory identified

the *CaCTR1* copper transporter by rescue of a *S. cerevisiae ctr1*Δ *ctr3*Δ mutant, and demonstrated that a *Cactr1*Δ/*Cactr1*Δ mutant has the same phenotypes as the *ctr1*Δ *ctr3*Δ mutant (Marvin *et al.*, 2003; Marvin *et al.*, 2004). Further analysis of these copper transporter genes revealed that the mouse Ctr1 gene is required for embryonic development, and that mice lacking the mCtr1 gene die in mid-gestation (Kuo *et al.*, 2001; Lee *et al.*, 2001). This surprising role for copper uptake in growth and development is also observed in the fruit fly *Drosophila melanogaster*, where two of the three copper uptake genes are required for normal growth and development of larvae (Turski & Thiele, 2007; Zhou *et al.*, 2003). In humans, hCtr1 has also been identified as a mechanism of uptake for the anti-cancer drug cisplatin and it is hoped that further investigation of this process will aid strategies to prevent cisplatin resistance (Ishida *et al.*, 2002; Lin *et al.*, 2002).

The Ctr family of copper transporter proteins is highly conserved from yeast to humans and the members of this family share common protein domains and motifs that contribute to copper uptake functions (Puig & Thiele, 2002). Comparison of Ctr proteins, illustrated in Figure 1.3, from *S. cerevisiae, C. albicans, S. pombe, A. thaliana*, humans and mice show that all proteins possess N-terminal methionine motifs, C-terminal cysteine/histidine motifs and three transmembrane domains, with the exception of *S. cerevisiae* Ctr3 which lacks methionine motifs (Marvin *et al.*, 2003; Puig & Thiele, 2002). Interestingly, the N-terminal domains of the Ctr proteins in species other than *S. cerevisiae* show the most homology to ScCtr1 and contain methionine motifs, whereas their C-terminal domains are more similar to ScCtr3 (Labbe *et al.*, 1999; Puig & Thiele, 2002). This gives the appearance of a fusion of the two *S. cerevisiae* Ctr proteins, suggesting that the functionally redundant *CTR1* and *CTR3* genes may be a result of whole genome duplication (Kellis *et al.*, 2004).

Copper-binding in the copper transporter proteins

Methionine, cysteine and histidine are all copper-ligands, and the mets and Cys/His motifs are both involved in copper-binding. The mets motifs contain three or five methionine residues in the sequence MXXM or MXM and are illustrated in Figure 1.3 (Marvin *et al.*, 2003; Puig & Thiele, 2002). There are between one and eight mets motifs in the N-terminal domain of each Ctr protein except Ctr3 (Marvin *et al.*, 2003; Puig & Thiele, 2002). Mets motifs with this sequence are also present in the extracellular domains of the bacterial copper binding proteins CopA and CopB from *Enterococcus hirae* and *Pseudomonas syringae* (Cha & Cooksey, 1991; Odermatt *et al.*, 1993). The function of the mets motifs as copper-binding sequences was confirmed by both chemical and genetic analyses (Jiang *et al.*, 2005; Puig *et al.*, 2002). A synthetic peptide of MXXMXXMX was shown to bind one Cu (I) ion with a

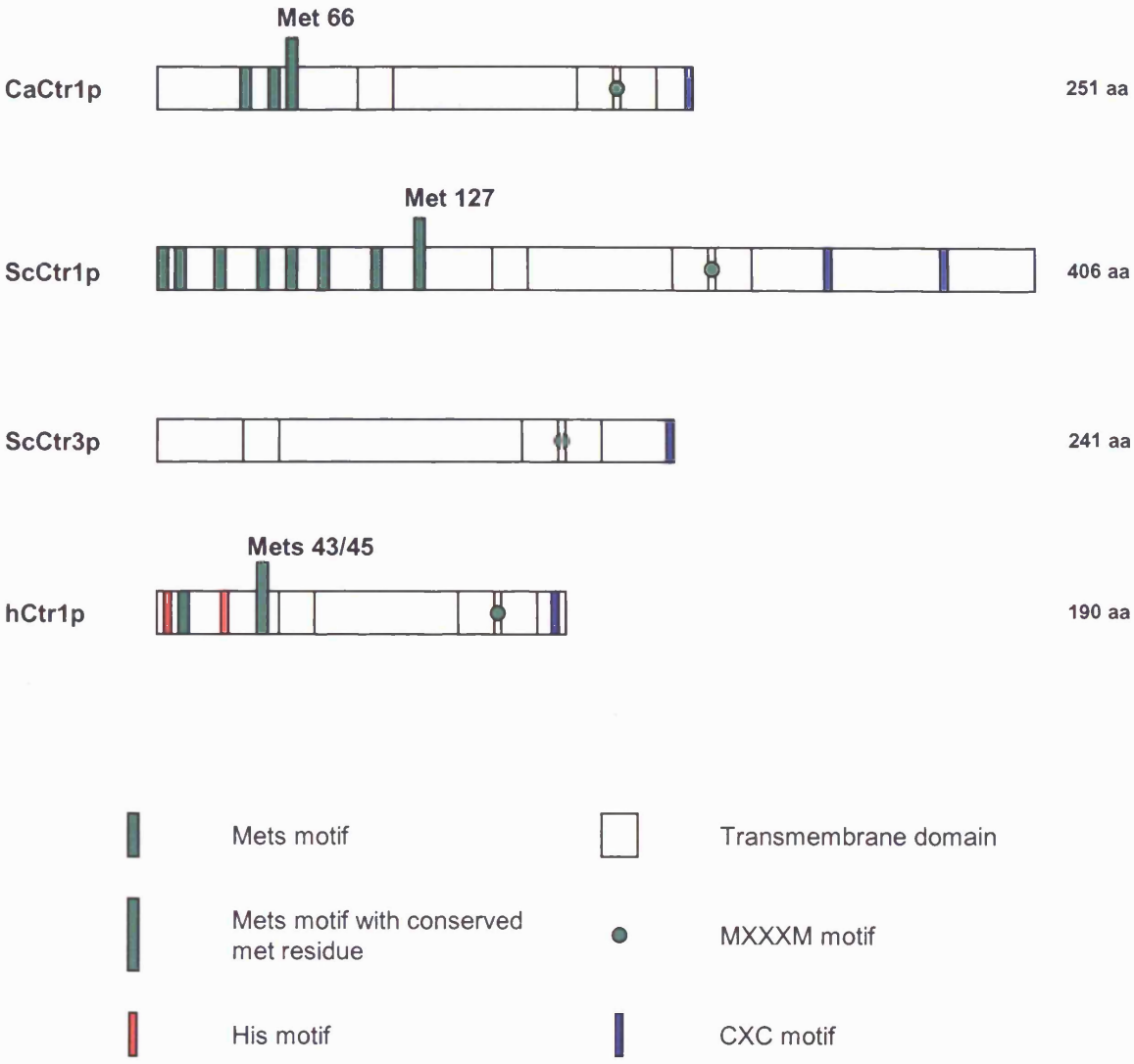


Figure 1.3 Comparison of copper transporter proteins.

The diagram indicates to scale the position of features conserved between Ctr proteins. The conserved positions of the last methionines of the Mets motifs is indicated. The proteins represented are *Candida albicans* Ctr1p, *Saccharomyces cerevisiae* Ctr1p, *Saccharomyces cerevisiae* Ctr3p and human Ctr1p. Adapted from Puig and Thiele, 2002.

dissociation constant of approximately 2.6 μM, which is equivalent to the affinity of the yeast high affinity copper uptake system (Jiang *et al.*, 2005). Substitution of any methionine in the peptide for the amino acid analogue norleucine reduced the copper-binding capacity to 90 μM and substitution of two of the methionines completely abolished copper binding (Jiang *et al.*, 2005). This shows that methionines alone are capable of binding copper without the assistance of the additional copper ligands histidine and cysteine.

Substitution of methionines in ScCtr1p has identified which residues are required for biologically relevant copper uptake (Puig *et al.*, 2002). Sequential removal of the N-terminal mets motifs decreased copper transport but did not abolish it, and only the last methionine of the mets motifs (Met-127) was essential for copper uptake (Puig *et al.*, 2002). The last two methionines in the hCtr1 N-terminal mets motifs were also essential for copper uptake (Puig *et al.*, 2002). In the case of both proteins, substitution of the neutral amino acid alanine abolished copper uptake, whereas the substitution of methionine for the copper ligands cysteine and histidine resulted in phenotypes similar to the wild type (Puig *et al.*, 2002). In contrast, work in our laboratory has shown that mutation of the last methionine of the mets motifs in CaCtr1p (Met-66) has no effect on copper uptake (R. Mason, personal communication). In place of methionine motifs, ScCtr3p contains a large number of CXXC and CC cysteine copper binding motifs, similar to those found in other yeast copper-binding proteins, and an N-terminal extracellular cysteine motif at amino acid position 16 which is required for effective copper transport (Pena *et al.*, 2000).

ScCtr1 and hCtr1 have both been localised to the plasma membrane, with topology showing that the N-terminal tail of the protein is extracellular and the C-terminal protein is intracellular, see Figure 1.4 (Dancis *et al.*, 1994b; Klomp *et al.*, 2003; Mercier *et al.*, 2006; Puig *et al.*, 2002). This suggests that the mets motifs are involved in binding copper prior to transport and that the Cys/His motifs are likely to be the point of transfer of intracellular copper to chaperones such as Atx1p (Lin *et al.*, 1997; Xiao *et al.*, 2004). Additional methionine-rich motifs with the sequence MXXXM are conserved between the second and third transmembrane domains of the Ctr proteins, and these may bind copper during transport across the plasma membrane (Marvin *et al.*, 2003; Puig & Thiele, 2002). Mutation of the methionine residues in this motif has revealed that both methionines are essential for copper transport by ScCtr3, hCtr1 and CaCtr1p (Puig *et al.*, 2002; Richards, 2004). However, only the second methionine residue in the mets motif is essential for copper transport in ScCtr1p and the first methionine does not contribute to the function of the protein (Puig *et al.*, 2002).

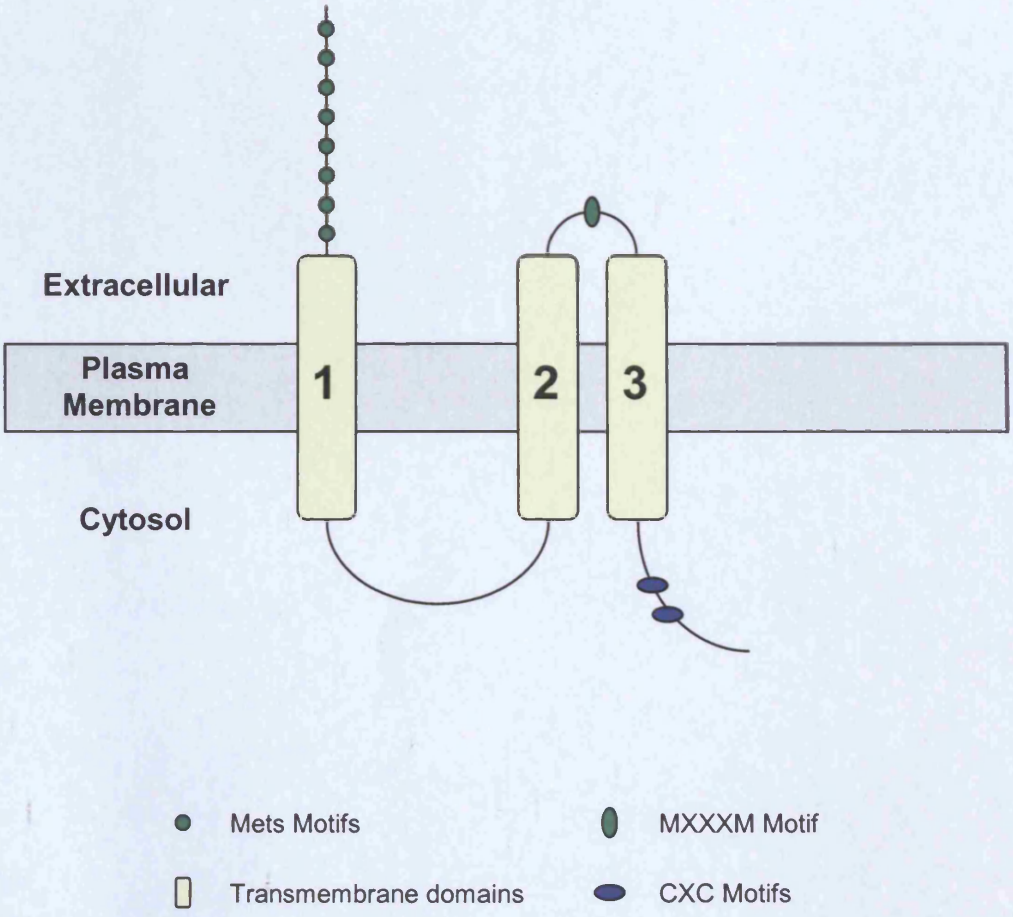


Figure 1.4 Topology of *S. cerevisiae* Ctr1p.

The diagram indicates the relative positions of the transmembrane domains and conserved motifs of Ctr1p. The N-terminus of the protein is extracellular and the C-terminus is intracellular. Adapted from Puig & Thiele, 2002.

In contrast to previously characterised yeast transporter proteins, each Ctr protein contains just three transmembrane domains suggesting that the Ctr proteins are likely to assemble as multimers to form a functional transporter complex (Nelissen *et al.*, 1997). In fact, co-immunoprecipitation experiments have shown that ScCtr1p, ScCtr3p and hCtr1 all form functional homotrimers at the plasma membrane, and this structure makes three MXXXM motifs available for copper binding (Aller & Unger, 2006; Pena *et al.*, 2000; Sinani *et al.*, 2007). There is still some debate about whether Ctr1 proteins facilitate copper uptake by forming a channel, or whether or not Ctr1 actively transports copper (Aller & Unger, 2006; Sinani *et al.*, 2007). The crystal structure of hCtr1 indicates that the homotrimeric proteins form a pore in the plasma membrane that resembles an ion channel, and that transport is driven by the copper gradient across the membrane (Aller & Unger, 2006). However, recent evidence suggests that a conformational change in ScCtr1p is associated with copper transport, and the conformational change is copper-responsive and specific (Sinani *et al.*, 2007). Interestingly, ScCtr1p-dependent uptake of cisplatin is not associated with a conformational change, and mutants defective for copper transport are still able to accumulate cisplatin, indicating that copper and cisplatin are transported using different mechanisms (Sinani *et al.*, 2007).

Intracellular copper transport

Intracellular copper levels must be carefully controlled to avoid the production of toxic reactive oxygen species. Copper levels in the cytosol of *S. cerevisiae* are extremely low with approximately one free copper ion per cell (Rae *et al.*, 1999). The level of free copper is low because the majority of copper is bound to copper chaperone proteins for delivery to various cuproproteins (Field *et al.*, 2002). The intracellular C-terminal domain of Ctr1p can bind four Cu^{+} ions, and Ctr1p containing mutations in the C-terminal cysteine residues binds less copper than the wild type protein (Xiao *et al.*, 2004). The sulphur atoms of cysteine residues can also directly interact with copper, supporting the hypothesis that copper is likely to be transferred to copper chaperones from the cysteine residues of Ctr1p (Xiao *et al.*, 2004).

A number of copper chaperones are used to transfer copper from Ctr1p to different cellular locations, for example, Atx1p is involved in the transfer of copper to the Golgi for the production of copper-loaded Fet3p (Klomp *et al.*, 1997; Lin *et al.*, 1997; Stearman *et al.*, 1996). Atx1p interacts with the P-type ATP-ase Ccc2p, which then transports copper from Atx1p into the Golgi where it is incorporated into Fet3p (Pufahl *et al.*, 1997; Yuan *et al.*, 1997). Deletion of *CCC2* abolishes high affinity ferrous iron uptake but deletion of *ATX1* only causes a partial reduction of iron uptake (Lin *et al.*, 1997), indicating that Ccc2p is

essential for copper-loading of Fet3p but that the transport of copper may be able to bypass the chaperone Atx1p in some cases. This theory is supported by evidence that Ctr1p is able to exchange copper ions with both Atx1p and Ccc2p *in vitro*, and copper may therefore be transferred directly from Ctr1p to Ccc2p (Xiao & Wedd, 2002; Xiao *et al.*, 2004). High affinity iron uptake in *C. albicans* also requires multicopper oxidase activity (Knight *et al.*, 2002), and the mechanism of copper-loading onto the *C. albicans* oxidase appears to be similar to *S. cerevisiae* due to the requirement of the *CCC2* homologue *CaCCC2* for ferrous iron transport (Weissman *et al.*, 2002).

Copper chaperones are also required for delivery of copper to other cupro-proteins, for example, the copper chaperone CCS delivers copper to the Cu/Zn superoxide dismutase Sod1p, and evidence suggests that CCS and Sod1p form a heterodimer to transfer copper between the two proteins (Casareno *et al.*, 1998; Lamb *et al.*, 2000; Lamb *et al.*, 2001). Cox17p delivers copper from Ctr1p to the mitochondria for incorporation into cytochrome *c* oxidase (Glerum *et al.*, 1996). However, transport of copper into the mitochondria is a complex process that is not fully understood at present, and involves a number of interations between copper-binding proteins in order to shuttle copper across the intramembrane space of the mitochondria, reviewed in (Carr & Winge, 2003).

Low affinity copper uptake

S. cerevisiae and *C. albicans* strains lacking the high affinity copper transporter genes *CTR1*, *CTR3* and *CaCTR1* are still viable and are able to grow in copper-replete conditions, suggesting the presence of a low affinity copper transporter on the cell surface (Dancis *et al.*, 1994b; Knight *et al.*, 1996; Marvin *et al.*, 2003). Low affinity copper uptake in *S. cerevisiae* is mediated by the low affinity ion transporter Fet4p, which can transport Fe^{2+}, Cd^{2+}, Zn^{2+} and Co^{2+} and Cu^{+} (Dix *et al.*, 1994; Hassett *et al.*, 2000). Hassett *et al.* also showed that while a *ctr1Δctr3Δfet4Δ* triple mutant did not have any detectable radioactive Cu^{+} uptake, it was still able to grow in copper replete conditions (Hassett *et al.*, 2000). The survival and growth of this triple mutant must therefore be due to an additional copper uptake system or the utilisation of intracellular copper stores. The low affinity metal transporter Smf1p can transport copper and overexpression of *SMF1* causes copper toxicity (Liu *et al.*, 1997). Smf1p also transports the divalent cations Fe^{2+} and Mn^{2+} and is therefore also likely to transport copper in its divalent form; this would explain why no radioactive Cu^{+} uptake was detected in the triple mutant (Liu *et al.*, 1997). This suggests that Smf1p may therefore be functional for copper uptake in oxidising conditions.

The role of the vacuole in copper homeostasis

Copper is stored in the vacuole and can be transported into the cytoplasm by Ctr2p in times of copper starvation (Rees *et al.*, 2004). Ctr2p is a high affinity copper transporter that requires reduction of Cu^{2+} in the vacuole by Fre6p before the reduced copper can be transported across the vacuolar membrane (Rees & Thiele, 2007; Singh *et al.*, 2007). Although a low affinity copper uptake system has not been described for *S. pombe*, vacuolar copper homeostasis is known to be important in copper starvation conditions and the Ctr6 protein exports copper from the vacuole (Bellemare *et al.*, 2002). A homologue of the *ScCTR2* gene (orf 19.4720) is present in the *C. albicans* genome sequence and is predicted to have a copper transport function analogous to *ScCTR2*, but this gene has not been experimentally characterised (Braun *et al.*, 2005). The vacuole is likely to play a role in copper homeostasis in *C. albicans* because disruption of the vacuole-associated protein Vac1p resulted in increased sensitivity to copper toxicity (Franke *et al.*, 2006).

1.7 Regulation of copper uptake

Copper uptake and metabolism must be carefully controlled and regulated to ensure that copper is available for essential cellular processes such as respiration but that copper levels in the cell do not become toxic. Regulation occurs at a number of levels including regulation of transcription, translation, and post-translational regulation. This section will focus on transcriptional regulation in response to extracellular copper levels in *C. albicans* and other fungi, but will also cover relevant aspects of post-transcriptional regulation.

Transcriptional responses to extracellular copper levels

The effects of extracellular copper levels have been studied using transcriptional profiling in both *S. cerevisiae* and *S. pombe* and these studies show that copper levels not only affect transcription of copper uptake and metabolism genes but appear to impact on respiration, metabolism of other metal ions, the oxidative stress response and a large number of genes with unknown functions (De Freitas *et al.*, 2004; Gross *et al.*, 2000; Rustici *et al.*, 2007; van Bakel *et al.*, 2005). These studies also showed that *YFR055w* and *YJL217w* are regulated by the copper-responsive transcription factor ScMac1p, but the regulation and function of these genes has not been investigated further (De Freitas *et al.*, 2004; Gross *et al.*, 2000).

Increased transcription of *AFT1* and the majority of Aft1p-regulated genes in low copper conditions revealed a clear link between the transcriptional response to copper and iron levels in *S. cerevisiae* (De Freitas *et al.*, 2004; Gross *et al.*, 2000; van Bakel *et al.*, 2005). Transcription of genes encoding copper- and iron-containing proteins, e.g. *CYC1* and *LYS4,*

was also decreased (De Freitas *et al.*, 2004; Gross *et al.*, 2000; van Bakel *et al.*, 2005). This evidence suggests that the copper starvation causes a secondary iron starvation which requires increased iron uptake and conservation of the existing iron within the cell. As a result of copper starvation transcription of the iron uptake genes *str1*$^+$ and *frp1*$^+$ was also induced in *S. pombe* indicating that iron may also be limiting in this organism in low copper conditions (Rustici *et al.*, 2007).

The copper-responsive yeast transcription factor family

The copper-responsive transcriptional activator ScMac1p was initially identified due to its similarity to the copper-responsive transcription factors Ace1p and Amt1p that regulate expression of metallothionein genes in *S. cerevisiae* and *C. glabrata* respectively (Jungmann *et al.*, 1993; Thorvaldsen *et al.*, 1993). These transcription factors share several functional domains in common which are illustrated in Figure 1.5, and these include N-terminal DNA binding domains and C-terminal copper-sensing and activation domains.

All the proteins have an N-terminal domain of approximately 40 amino acids, termed a copper-fist motif, which is highly conserved and is required for the DNA binding activities of Ace1, Amt1 and Mac1 (Dameron *et al.*, 1991; Farrell *et al.*, 1996; Joshi *et al.*, 1999). The copper-fist motif contains a binding region for a single zinc ion that consists of conserved cysteine and histidine residues (Farrell *et al.*, 1996). Direct binding of zinc ions has been demonstrated for Amt1p and Ace1p, but the highly conserved nature of the copper fist strongly suggests that all of the proteins are able to bind zinc in this position (Farrell *et al.*, 1996; Thorvaldsen *et al.*, 1994). The copper fist also contains a (R/K)GRP DNA binding motif at the C-terminal end of the copper fist domain that binds to the minor groove of DNA (Koch & Thiele, 1996).

Another requirement for DNA binding activity is the ability to form homodimers (Serpe *et al.*, 1999). ScMac1p binds to target promoters as a homodimer and the level of dimerisation correlates to activation activity, supporting the evidence that the CuREs work in synergy due to the increased protein molecules bound to the DNA (Jensen *et al.*, 1998; Joshi *et al.*, 1999). Residues 388-406 in the predicted D-helix are required for the formation of the ScMac1p homodimer, by protein-protein interactions, and deletion or mutation of this domain prevents the rescue of a *mac1*Δ mutant (Serpe *et al.*, 1999). This indicates that dimerisation is essential for the function of ScMac1p *in vivo*.

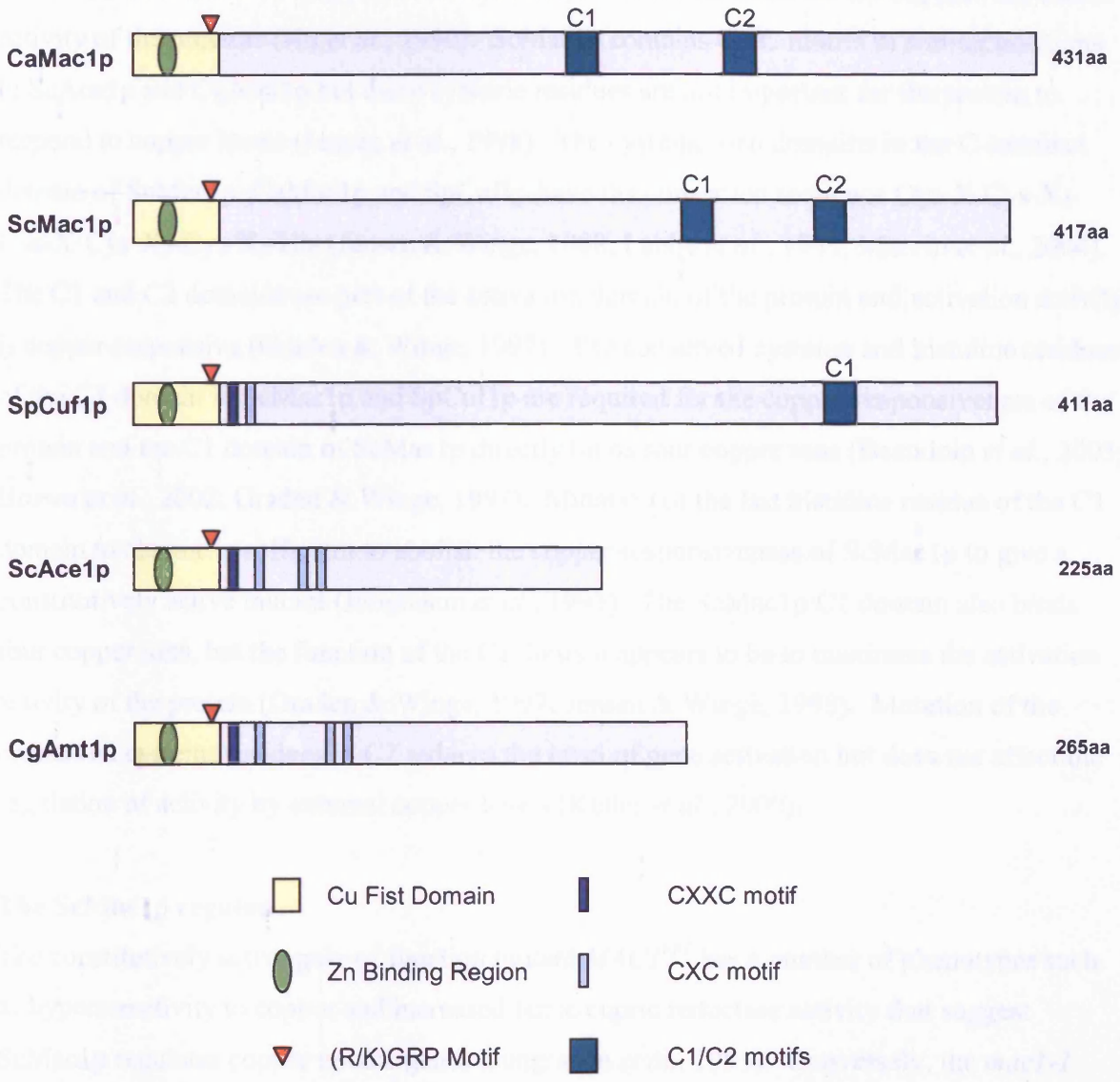


Figure 1.5 Comparison of copper-responsive transcription factors from yeast.

The diagram indicates to scale the position of features conserved in the yeast copper-responsive transcription factors. The proteins represented are *Candida albicans* Mac1p, *Saccharomyces cerevisiae* Mac1p, *Schizosaccharomyces pombe* Cuf1p, *Saccharomyces cerevisiae* Ace1p and *Candida glabrata* Amt1p. Adapted from Rutherford & Bird (2004).

The structure of ScMac1p differs from ScAce1p and CgAmt1p in the cysteine-rich copper-binding regions of the protein. ScAce1p and CgAmt1p contain one CXC and three CXXC motifs within the amino terminal 100 amino acids that are essential for the copper-responsive activity of the proteins (Hu *et al.*, 1990). ScMac1p contains CXC motifs in similar positions to ScAce1p and CgAmt1p but these cysteine residues are not important for the protein to respond to copper levels (Jensen *et al.*, 1998). The cysteine-rich domains in the C-terminal domain of ScMac1p, CaMac1p and SpCuf1p have the conserved sequence Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys-X_2-His (Jensen & Winge, 1998; Labbe *et al.*, 1999; Marvin *et al.*, 2004). The C1 and C2 domains are part of the activation domain of the protein and activation activity is copper-responsive (Graden & Winge, 1997). The conserved cysteine and histidine residues of the C1 domain in ScMac1p and SpCuf1p are required for the copper-responsiveness of the protein and the C1 domain of ScMac1p directly binds four copper ions (Beaudoin *et al.*, 2003; Brown *et al.*, 2002; Graden & Winge, 1997). Mutation of the last histidine residue of the C1 domain to alanine is sufficient to abolish the copper-responsiveness of ScMac1p to give a constitutively active mutant (Jungmann *et al.*, 1993). The ScMac1p C2 domain also binds four copper ions, but the function of the C2 domain appears to be to maximise the activation activity of the protein (Graden & Winge, 1997; Jensen & Winge, 1998). Mutation of the conserved cysteine residues in C2 reduces the level of gene activation but does not affect the regulation of activity by external copper levels (Keller *et al.*, 2000).

The ScMac1p regulon

The constitutively active gain of function mutant *MAC1*^{up1} has a number of phenotypes such as hypersensitivity to copper and increased ferric/cupric reductase activity that suggest ScMac1p regulates copper uptake genes (Jungmann *et al.*, 1993). Conversely, the *mac1-1* mutant is respiratory deficient, sensitive to oxidative stress, grows slowly in low copper media and has low levels of ferric/cupric reductase activity (Jungmann *et al.*, 1993).

The first gene to be identified in the ScMac1p regulon was the ferric/cupric reductase gene *FRE1* due to the similar phenotypes of the *mac1-1* and *fre1*Δ mutants which include reduced ferric and cupric reductase activity, slow growth in low copper and iron media, respiratory deficiency and hypersensitivity to oxidative stress (Jungmann *et al.*, 1993). Transcription of *FRE1* is increased in copper starvation conditions, but no transcript was detected in *mac1-1* mutants (Jungmann *et al.*, 1993). Further analysis showed that activation activity of ScMac1p is increased in low copper levels, confirming that ScMac1p is a transcriptional activator rather than a repressor (Georgatsou *et al.*, 1997). The ScMac1p regulon was expanded when *ScMAC1* was shown to be required for copper-regulated transcription of the copper

transporter genes *CTR1* and *CTR3* and the ferric reductase-like gene *FRE7* (Labbe *et al.*, 1997; Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). Comparison of their promoters revealed a common promoter element with the sequence TTTGC(T/G)CA which resembles the promoter element recognised by ScAce1p and CgAmt1p (Labbe *et al.*, 1997; Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). The promoter element was therefore named the copper response element, or CuRE (Yamaguchi-Iwai *et al.*, 1997). ScMac1p binds to this promoter element in the absence of copper and two copies of the sequence in the promoter are required for effective copper regulation of transcription (Heredia *et al.*, 2001; Labbe *et al.*, 1997; Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). The level of activation is affected by the strength of binding to the CuRE, and increased DNA binding and activation activity is seen in CuREs preceded by TA compared with TT, due to increased binding to TA by ScMac1p (Joshi *et al.*, 1999). This sequence difference may affect binding of the DNA in the minor groove by the RGRP motif of ScMac1p, as this motif binds the TTT sequence of the CuRE (Jamison McDaniels *et al.*, 1999).

The presence of CuREs was used as one of the criteria for the identification of additional members of the ScMac1p regulon using microarray technology (De Freitas *et al.*, 2004; Gross *et al.*, 2000). Two transcriptional profiling studies identified a putative cystathione γ-lyase gene (*YFR055w*) and another gene of unknown function (*YJL217w*) as potential target genes for regulation by ScMac1p (De Freitas *et al.*, 2004; Gross *et al.*, 2000). However, there is only one CuRE in the promoters of these genes and further investigation into the function or regulation has not been carried out, leaving some uncertainty as to whether they are in fact transcriptionally activated by ScMac1p.

Post-translational regulation of copper uptake in *S. cerevisiae*

High affinity copper uptake is tightly controlled at the transcriptional level, but additional controls are in place at the post-translational level to prevent existing Ctr1p from transporting potentially toxic levels of copper into the cell. The Ctr1p copper transporter protein is internalized and degraded at copper concentrations of 10 μM and above (Ooi *et al.*, 1996). The ScMac1 protein is also required for degradation of Ctr1p in high copper levels but it is not known how ScMac1p regulates the degradation of Ctr1p (Yonkovich *et al.*, 2002). It is possible that ScMac1p activates components of this protein degradation pathway. A gene encoding a protein with no known function, *YJL217w,* was previously identified as a potential ScMac1p target and could be involved in this process (Gross *et al.*, 2000). ScCtr3p is not degraded in response to copper levels, and the maintenance of ScCtr3p at the plasma

membrane may prevent copper starvation in the event of a sudden decrease in extracellular copper levels (Pena *et al.*, 2000).

Although transcription of the *ScMAC1* gene is constitutive, ScMac1p is degraded in response to potentially toxic copper concentrations of greater than 10 μM (Yonkovich *et al.*, 2002; Zhu *et al.*, 1998). ScMac1p target genes are not transcribed at copper concentrations of 1 μM or above (Zhu *et al.*, 1998). This is because an intramolecular interaction between the DNA binding and activation domains of ScMac1p takes place in the presence of moderate copper levels, of 1 μM or higher, that blocks the DNA binding and transactivation activities of the protein (Jensen & Winge, 1998). Therefore transcription of ScMac1p target genes is rapidly prevented in the presence of copper at 1-10 μM, and the protein is only degraded if the copper level exceeds this range.

Copper-dependent DNA binding of ScMac1p is not observed in proteins expressed in *E. coli* or *in vitro* transcription and translation systems, but overexpression of ScMac1p in yeast showed that DNA binding could be repressed by the addition of as little as 1 μM $CuSO_4$ (Heredia *et al.*, 2001; Jensen *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). These two different mechanisms of regulating ScMac1p are thought to represent different responses to toxic and nutritionally useful copper levels. Analysis of full length proteins expressed in *S. cerevisiae* also revealed that native ScMac1p is phosphorylated and that phosphorylation was required for DNA binding in yeast (Heredia *et al.*, 2001).

Copper-responsive transcriptional regulation in *C. albicans*

Previous work in our laboratory identified a functional homologue of *ScMAC1* in *C. albicans* and showed that a *camac1Δ/ camac1Δ* mutant demonstrates similar phenotypes to the *mac1Δ* mutant including slow growth in low copper and iron, respiratory deficiency and sensitivity to oxidative stress (Marvin *et al.*, 2004; Mason, 2006). *CaMAC1* is also required for transcription of the copper transporter gene *CaCTR1* in low copper conditions (Marvin *et al.*, 2004). Heterologous expression of *CaCTR1* in *S. cerevisiae* requires the presence of the *ScMAC1* gene and there are three CuRE sequences in the *CaCTR1* promoter (Marvin *et al.*, 2004). During the preparation of this thesis, Levitin *et al.* demonstrated that a CuRE sequence was involved in transcription of *CaFRE7*, the closest *C. albicans* homologue of *ScFRE7*. This suggested that CaMac1p was likely to regulate *CaFRE7* transcription, but the roles of CaMac1p and copper-regulation were not investigated in this study (Levitin & Whiteway, 2007). This evidence indicates that the CaMac1p activator is the true functional homologue of ScMac1p and appears to use a similar mechanism to activate its target genes.

CaMac1p shares conserved motifs with the copper-responsive transcription factor family and, like ScMac1p, has two C-terminal cysteine-rich domains. The constitutively active gain of function mutant *MAC1*^{up1} contains a H279Q mutation in the last histidine of the C1 domain rendering it unresponsive to copper levels, whereas an equivalent H211Q mutation in CaMac1p did not have any effect on the function of CaMac1p (Jungmann *et al.*, 1993; Thomas, 2003). In addition, mutation of the terminal histidine of CaMac1p C2 to glutamine did not have any effect, suggesting that the histidine residues in the C1/C2 domains may not participate in copper binding and that CaMac1p coordinates copper in a different way to ScMac1p (Thomas, 2003).

1.8 Background to the project

The aims of this project were to identify additional genes regulated by CaMac1p and to further investigate how CaMac1p regulates expression. Previous work in our laboratory had shown that the *CaCTR1* promoter contains three sequences identical to the *S. cerevisiae* CuREs and that heterologous expression of *CaCTR1* in *S. cerevisiae* requires the presence of *ScMAC1* (Marvin *et al.*, 2004). This evidence indicated that CaMac1p functions in a similar way to ScMac1p to activate expression of *CaCTR1* in low copper conditions. However, mutation in our laboratory of key histidine residues in CaMac1p, that are conserved in ScMac1p and important for copper sensing, failed to produce a constitutive gain-of-function mutant similar to *S. cerevisiae MAC1*^{up1} (Jungmann *et al.*, 1993; Thomas, 2003). Although many of the genes involved in iron uptake in *S. cerevisiae* and *C. albicans* are similar, the regulation of iron homeostasis is actually facilitated by different transcription factors. This suggests that copper-dependent regulation in the opportunistic pathogen *C. albicans* may also be different from copper regulation in the model organism *S. cerevisiae.*

1.9 List of objectives

- Determine whether the CuREs in the *CaCTR1* promoter are involved in copper-dependent regulation of *CaCTR1* expression.
- Identify additional genes in the *C. albicans* genome with two or more CuRE sequences in their promoters
- Determine whether genes with two or more CuREs in their promoters are regulated in response to copper and CaMac1p
- Investigate the importance of CuRE sequences in the promoters of additional genes regulated by CaMac1p

Chapter 2

Materials and Methods

2.1 Strains used during this study

Bacterial strains

E. coli strain XL10 Gold® purchased from Stratagene was used for all bacterial work.

Genotype:

(Tet^{R}Δ *(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F'proAB lac^{q}ZΔM15* Tn*10* (Tet^R) Amy Cam^R])

***Candida albicans* strains**

See Table 2.1 for all strains used in this study

2.2 Media and growth conditions

Growth of *E. coli* strains

E. coli were grown in Luria Bertani (LB) medium at 37°C overnight with liquid cultures undergoing shaking at 200 rpm. The components of LB were 1 % (w/v) Bacto-tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) sodium chloride, adjusted to pH 7.2 with sodium hydroxide. Solid media contained 2 % (w/v) Bactoagar and all media components were purchased from Oxoid. Ampicillin was used a selective agent at a final concentration of 100 μg ml^{-1}.

Growth of *C. albicans* strains

All strains were grown at 30°C, with liquid cultures undergoing shaking at 200 rpm.

Yeast Peptone Glucose Media (YPD)

C. albicans strains were routinely grown in Yeast Peptone Glucose Media made with 1 % (w/v) yeast extract, 2 % (w/v) Bactopeptone, 2 % (v/v) glucose and 50 μg ml^{-1} uridine. Solid media also contained 2 % (w/v) Bactoagar and all media components were purchased from Oxoid.

Synthetic Defined Media (SD)

Synthetic Defined Media contained 2 % (w/v) Bactoagar, 0.67 % (w/v) yeast nitrogen base without amino acids (Bio 101), 2 % (v/v) glucose and was supplemented with amino acids at

Table 2.1 *Candida albicans* strains used during this study

Strain	Genotype	Reference
SC5314	Clinical isolate	(Gillum *et al.*, 1984)
BWP17	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	(Wilson *et al.*, 1999)
MEM-m2	As BWP17 but *mac1*Δ::*URA3/mac1*Δ::*ARG4; his1::hisG/his1::hisG::HIS1*	(Marvin *et al.*, 2004)
MEM-c3	As BWP17 but *ctr1*Δ::*URA3/ctr1*Δ::*ARG4*; *his1::hisG/his1::hisG::HIS1*	(Marvin *et al.*, 2003)
AWP1	BWP17 with *RPS10*::plac-poly/*RPS10*	This study
AWC1	BWP17 with *RPS10*::pAWC1/*RPS10*	This study
AWC1.1	MEM-m2 with *RPS10*::pAWC1/*RPS10*	This study
AWC2	BWP17 with *RPS10*::pAWC2/*RPS10*	This study
AWC3	BWP17 with *RPS10*::pAWC3/*RPS10*	This study
AWC4	BWP17 with *RPS10*::pAWC4/*RPS10*	This study
AWC5	BWP17 with *RPS10*::pAWC5/*RPS10*	This study
AWC6	BWP17 with *RPS10*::pAWC6/*RPS10*	This study
AWC7	BWP17 with *RPS10*::pAWC7/*RPS10*	This study
AWC8	BWP17 with *RPS10*::pAWC8/*RPS10*	This study
AWC9	BWP17 with *RPS10*::pAWC9/*RPS10*	This study
AWC10	BWP17 with *RPS10*::pAWC10/*RPS10*	This study
AWC11	BWP17 with *RPS10*::pAWC11/*RPS10*	This study
AWM1	BWP17 with *RPS10*::pAWM1 /*RPS10*	This study
AWM1.1	MEM-m2 with *RPS10*::pAWM1/*RPS10*	This study
AWM2	BWP17 with *RPS10*::pAWM2/*RPS10*	This study
AWF1	BWP17 with *RPS10*::pAWF1/*RPS10*	This study
AWF1.1	MEM-m2 with *RPS10*::pAWF1/*RPS10*	This study
AWF2	BWP17 with *RPS10*::pAWF2/*RPS10*	This study
AWF3	BWP17 with *RPS10*::pAWF3/*RPS10*	This study
AWF4	BWP17 with *RPS10*::pAWF4/*RPS10*	This study

the concentrations shown in Table 2.2. SD supplemented with 1 mg ml^{-1} 5-FOA and 50 $\mu g\ ml^{-1}$ uridine was used to counterselect for *CaURA3*.

Minimal Defined Media (MD)

Minimal Defined media is based on Wickerham's media (Wickerham, 1946) and includes some of the modifications made by David Eide and coworkers (Eide & Guarente, 1992). The medium contains 10 % (v/v) salt and trace solution, 0.1 % (v/v) vitamin solution, 2 % (v/v) glucose, 7 mM calcium chloride and 20 mM tri-sodium citrate (pH 4.2). The components of salt and trace solution and vitamin solution are detailed in Tables 2.3 and 2.4 respectively. All MD media was supplemented with amino acids at the concentrations shown in Table 2.2.

High copper medium

MD with 50 μM Bathocuproinedisulfonic acid (BCS), 100 μM $FeCl_3$ and 100 μM $CuCl_2$.

Low copper medium

MD with 50 μM Bathocuproinedisulfonic acid (BCS) and 100 μM $FeCl_3$.

High iron medium

MD with 50 μM Bathophenanthrolinedisulfonic acid (BPS), 100 μM $CuCl_2$ and 100 μM $FeCl_3$.

Low iron medium

MD with 50 μM Bathophenanthrolinedisulfonic acid (BPS) and 100 μM $CuCl_2$.

2.3 Preparation and manipulation of nucleic acids

Preparation of plasmid DNA

Plasmids used in this study are listed in Table 2.5. The plac-poly reporter plasmid (Brown et al., personal communication) is illustrated in Figure 2.1. Plasmid DNA was prepared using the Qiaprep Spin Mini Prep kit (Qiagen), following the manufacturer's instructions.

Preparation of *C. albicans* genomic DNA

An overnight culture in 10 ml of YPD was harvested by centrifugation at 4000 rpm for 5 minutes. After the supernatant was removed, the pellet was resuspended in 0.5 ml of sterile water and transferred to a screw cap microcentrifuge tube. The resuspended cells were centrifuged at 13000 rpm for 1 minutes and the supernatant discarded. The pellet was disrupted by vortexing for 10 seconds and was resuspended in 200 μl of breaking buffer (2 % (v/v) Triton X, 1 % (v/v) SDS, 100 mM NaCl, 10 mM TrisCl pH 8.0, 1 mM EDTA, pH 8.0).

Table 2.2 Amino acid supplements

Amino acid	Stock concentration	Final concentration
Arginine	4 mg ml^{-1}	20 μg ml^{-1}
Histidine	8 mg ml^{-1}	20 μg ml^{-1}
Uridine	50 mg ml^{-1}	50 μg ml^{-1}

Table 2.3 Salt and trace solution

Component	Stock concentration	Final concentration
Ammonium sulphate	75.7 mM	7.57 mM
Potassium dihydrogen orthophosphate	50.2 mM	5.02 mM
Di-potassium hydrogen orthophosphate	9.2 mM	0.92 mM
Magnesium sulphate	20.3 mM	2.03 mM
Sodium chloride	17.1 mM	1.71 mM
Boric acid	1.62 μM	162 nM
Potassium iodide	0.6μM	60 nM
Zinc sulphate	2.44 μM	244 nM

Table 2.4 Vitamin solution

Component	Stock concentration	Final concentration
d-biotin	8.19 μM	8.19 nM
Thiamine hydrochloride	1.19 mM	1.19 μM
Pyridoxine hydrochloride	1.95 mM	1.95 μM
Myo-inositol	11 mM	11 μM
d-pantothenic acid calcium salt	0.84 mM	0.84 μM

Table 2.5 Plasmids used during this study

Plasmid	Genotype	Reference
plac-poly	*ori; amp^R; CaRPS10; CaURA3; LacZ*	Brown *et al.*, unpublished
pAWC1	plac-poly with 776 bp *CaCTR1* promoter	This study
pAWC2	pAWC1 with point mutation in CuRE 1	This study
pAWC3	pAWC1 with point mutation in CuRE 2	This study
pAWC4	pAWC1 with point mutation in CuRE 3	This study
pAWC5	pAWC1 with point mutations in CuREs 1 & 2	This study
pAWC6	pAWC1 with point mutations in CuREs1 & 3	This study
pAWC7	pAWC1 with point mutations in CuREs 2 & 3	This study
pAWC8	pAWC1 with point mutations in CuREs 1, 2 & 3	This study
pAWC9	plac-poly with 387 bp *CaCTR1* promoter	This study
pAWC10	plac-poly with 263 bp *CaCTR1* promoter	This study
pAWC11	plac-poly with 202 bp *CaCTR1* promoter	This study
pAWM1	placpoly with 683 bp *CaMAC1* promoter	This study
pAWM2	pAWM1 with point mutation at CuRE	This study
pAWF1	placpoly with 796 bp *FRE12* promoter	This study
pAWF2	pAWF1 with point mutation in CuRE 1	This study
pAWF3	pAWF1 with point mutation in CuRE 2	This study
pAWF4	pAWF1 with point mutations in CuREs 1 & 2	This study

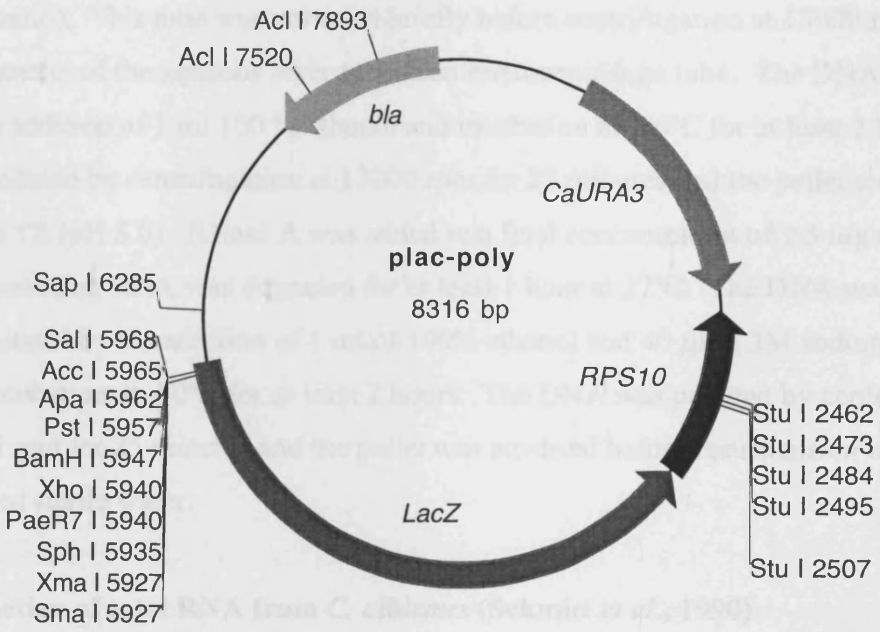


Figure 2.1 Plasmid map of plac-poly

The plasmid plac-poly contains a *LacZ* reporter gene derived from *Streptococcus thermophilus*. It also contains a gene encoding ampicillin resistance for selection and maintenance in *E. coli*. The *CaURA3* gene is used as an auxotrophic marker for selection of transformants in *C. albicans*. The *RPS10* gene is used for targeted integration into the genome of *C. albicans* following restriction digestion with *Stu*I.

An approximately equal volume (200 µl) of acid washed glass beads (Sigma) were added to the tube, along with 200 µl phenol:chloroform:isoamylalchol (25:24:1 ratio). The tubes were vortexed for 7 minutes using a multi-tube vortexer and 200 µl of TE was added (10 mM TrisCl pH 8.0, 1 mM EDTA pH 8.0). After centrifugation at 13000 rpm for 5 minutes, the aqueous layer was transferred to a fresh tube containing 500 µl chloroform:isoamylalcohol (24:1 ratio). This tube was vortexed briefly before centrifugation at 13000 rpm for 5 minutes and transfer of the aqueous layer to a clean microcentrifuge tube. The DNA was precipitated by the addition of 1 ml 100 % ethanol and incubation at -20°C for at least 2 hours. The DNA was pelleted by centrifugation at 13000 rpm for 25 minutes and the pellet was resuspended in 0.4 ml TE (pH 8.0). RNase A was added to a final concentration of 2.5 mg ml^{-1} and the contaminating RNA was degraded for at least 1 hour at 37°C. The DNA was again precipitated by the addition of 1 ml of 100% ethanol and 40 µl of 3M sodium acetate (pH 5.2) and incubation at -20°C for at least 2 hours. The DNA was pelleted by centrifugation at 13000 rpm for 25 minutes and the pellet was air-dried before resuspension in 100 µl deionised distilled sterile water.

Extraction of total RNA from *C. albicans* (Schmitt *et al.*, 1990)

A single colony was used to inoculate 10 ml of YPD and the culture was incubated for approximately six hours at 30°C with shaking. The culture was harvested by centrifugation at 4000 rpm for 5 minutes and the pellet was washed twice in sterile distilled water and resuspended in a final volume of 1 ml of sterile distilled water. The optical density of the suspension at 600 nm was determined to estimate the cell density based on previous growth curve experiments. A culture containing approximately $3x10^4$ cells ml^{-1} in 50 ml of MD containing high copper and high iron was grown overnight at 30°C with shaking. The next morning the cultures were harvested, washed and resuspended in a final volume of 1 ml sterile distilled water as previously. The same overnight culture was used to inoculate flasks containing 100 ml of MD with different supplements to a cell density of $2x10^6$ cells ml^{-1}. This culture was grown at 30°C with shaking for 5 hours until it reached exponential growth and a cell density of approximately $1x10^7$ cells ml^{-1} and the culture was then harvested for RNA extraction.

Exponentially growing cultures were harvested by centrifugation at 4000 rpm for 5 minutes, washed in 1 ml of sterile distilled water and resuspended in 400 µl RNase free AE buffer (50 mM sodium acetate pH 5.3, 10 mM EDTA). 80 µl of 10 % (w/v) SDS was added and vortexed for 30 seconds. An equal amount (480 µl) of phenol equilibriated with AE buffer was added and vortexed for 30 seconds. Samples were incubated at 65°C for 4 minutes before

snap-freezing in dry ice for 3 minutes. This process was repeated a further three times with a final incubation at 65°C for 4 minutes. The samples were centrifuged at 13000 rpm for 5 minutes and the aqueous phase was transferred to a fresh tube containing 500 μl of phenol:chloroform:isoamylalcohol (25:24:1 ratio). The tubes were vortexed briefly and centrifuged at 13000 rpm for 10 minutes at 4°C. This step was repeated and the aqueous phase transferred to a clean tube. The RNA was precipitated by adding 40 μl of 3 M sodium acetate and 2 volumes of 100% ethanol and incubation overnight at -80°C. The samples were centrifuged at 13000 rpm for 25 minutes at 4°C and washed with 500 μl of 80 % ethanol. After centrifugation at 13000 rpm for 25 minutes at 4°C, the pellets were air dried and resuspended in 50 μl DEPC treated water. DEPC (Di-ethyl-pyrocarbonate) treatment was performed by adding 0.1 % (v/v) DEPC to the solution overnight and then autoclaving the solution to destroy the DEPC for a minimum of 15 minutes at 115 °C and pressure of 15 pounds per square inch. All RNase free solutions used were rendered RNase free by DEPC treatment or were made with DEPC treated water.

Measurement of nucleic acid concentrations

Nucleic acid concentrations were calculated from spectrophotometer readings based on the following values.

1 OD_{260} unit = 50 μg of double stranded DNA

1 OD_{260} unit = 33 μg of single stranded DNA

1 OD_{260} unit = 40 μg of RNA

Agarose gel electrophoresis of DNA

DNA was separated and visualised using gels made from agarose (Seakem LE agarose, Cambrex) dissolved in 1 x TAE (Tris acetate electrophoresis) buffer with 25 μg ml^{-1} of ethidium bromide. Loading buffer (15 % (w/v) Ficoll 400, 0.06 % (w/v) bromophenol blue, 0.06 % (w/v) xylene cyanol FF, 30 mM EDTA) was added to DNA samples at a 1/6 dilution. Samples were loaded onto the gel before electrophoresis in 1 x TAE buffer. Electrophoresis was typically performed at 10 volts per centimetre of gel and gels were visualised using a UV transilluminator.

Denaturing agarose gel electrophoresis of RNA

Denaturing gels for RNA were composed of 1.5 % (w/v) agarose dissolved in 1 x MOPS (0.2 M MOPS, 50 mM sodium acetate, 1 mM EDTA) and 5 % formaldehyde in DEPC treated water. RNA samples (typically 30 μg RNA in a 5 μl volume) were treated prior to electrophoresis with 10 μl of deionised formamide, 2 μl of 10 x MOPS and 3.5 μl of 40 %

formaldehyde. They were incubated for 10 minutes at 65°C and chilled on ice for 5 minutes. Ethidium bromide to a final concentration of 4 μg ml^{-1} and 2.5 μl of 10 x loading buffer were added to the RNA samples before loading onto the gel. Electrophoresis was carried out in 1 x MOPS for 3 hours at 100 volts, and visualised using a UV transilluminator.

Restriction enzyme digestion of DNA

Restriction enzymes were purchased from New England Biolabs Ltd. and all digestions were carried out using the buffers supplied and following the manufacturer's instructions. *C. albicans* genomic DNA was typically digested overnight using 10 units of restriction enzyme for every 1 μg of DNA. Plasmid DNA and PCR products were typically digested for 3 hours using 10 units of restriction enzyme for every 5 μg of DNA.

Ligation of DNA

Phosphate groups were removed from vectors prior to ligation using Antarctic Phosphatase (New England Biolabs Ltd.) using the buffer supplied and following the manufacturer's instructions. Vector and insert DNAs were quantified and a molar ratio of insert:vector of 3:1 was used in the ligation, typically using 50 ng of vector. Ligations were performed in a total volume of either 10 μl or 20 μl. The vector, insert and sterile distilled water were incubated at 65°C for 5 minutes and then chilled on ice for 5 minutes. 400 units of T4 DNA Ligase (New England Biolabs Ltd.) and 1 x T4 DNA Ligase reaction buffer were then added before incubation overnight at 16°C.

2.4 Transformation procedures

Calcium chloride transformation of *E. coli* (Mandel & Higa, 1970)

An overnight culture of *E. coli* in LB was diluted one hundred fold in fresh LB and grown for 2-3 hours until exponential phase was reached (OD_{600} of approximately 0.5). The cells were chilled on ice for 10 minutes before harvesting by centrifugation at 4000 rpm for 5 minutes at 4°C. The pellets were washed three times in 0.5 volumes of ice-cold 100 mM calcium chloride with a final resuspension in 0.5 volumes of ice-cold 100 mM calcium chloride. The cells were incubated on ice for 30-60 minutes. Cells were harvested by centrifugation at 4000 rpm for 5 minutes at 4°C and the pellets resuspended in 0.05 volumes of ice-cold 100 mM calcium chloride. The competent cells were either transformed immediately or stored at -80°C after adding glycerol to a final concentration of 15 % and snap freezing on dry ice.

Transforming DNA was incubated with 200 μl of competent cells on ice for 30 minutes. The transformation samples were then heat shocked at 42°C for 2 minutes before adding 800 μl LB and incubation at 37°C for 60-90 minutes. The cells were harvested by centrifugation at 13000 rpm for 1 minute at room temperature and the pellets resuspended in 150 μl fresh LB. The transformations were spread onto LA plates containing 100 μg ml^{-1} ampicillin and incubated overnight at 37°C.

Lithium acetate transformation of *C. albicans*, adapted from (Wilson *et al.*, 1999)

An overnight culture of the *C. albicans* strain to be transformed in 10 ml of YPD was used to inoculate 50 ml of fresh YPD to give a concentration of approximately 1 x 10^6 cells ml^{-1}. This culture was grown for 4-5 hours to a concentration of approximately 2 x 10^7 cells ml^{-1}. The cells were harvested by centrifugation at 4000 rpm for 5 minutes at room temperature and washed with 10 ml of sterile distilled water. The pellet was resuspended in 0.5 ml LATE solution (0.1 M lithium acetate, 10 mM Tris-Cl, 1 mM EDTA). 100 μl of competent cells were added to the transforming DNA and 50 μg of single stranded salmon sperm DNA. The transformation mixture was incubated at room temperature for 30 minutes before the addition of 700 μl PLATE solution (40 % PEG 3500, 0.1 M lithium acetate, 10 mM Tris-Cl, 1 mM EDTA) and incubation overnight at room temperature. The next day, transformations were heat shocked at 42°C for 1 hour and harvested at 3000 rpm for 3 minutes. The pellets were washed in 1 ml of sterile distilled water and resuspended in 100 μl of fresh sterile distilled water. This was spread onto selective SD plates and incubated at 30°C for 3-7 days.

2.5 DNA sequencing and polymerase chain reaction

DNA sequencing

The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was used to sequence plasmid DNA. The terminator pre-mix was diluted 1/8 with 5 x Sequencing Buffer (Applied Biosystems) before use. Sequencing reactions comprised of 4 μl diluted terminator pre-mix, 100 ng plasmid template DNA and 2 pmoles of primer in a total volume of 10 μl distilled deionised water. Primers used for sequencing are shown in Table 2.6. If the melting temperature of the primer was below 60°C the reaction involved one cycle at 94°C for 0.3 minutes followed by 30 cycles of 96°C for 0.1 minutes, 50°C for 0.05 minutes and 60°C for 4 minutes. If the melting temperature of the primer was 60-70°C the reaction involved one cycle at 94°C for 5 minutes followed by 30 cycles of 96°C for 0.1 minutes and 60°C for 4 minutes. Completed reactions were purified using Performa® Gel Filtration Cartridges

(Edge BioSystems) or DyeEx 2.0 Spin Kit (Qiagen). The purified reactions were analysed by the University of Leicester's Protein and Nucleic Acid Chemistry Laboratory using an Applied Biosystems 3730 sequencer.

Polymerase chain reactions

Products of polymerase chain reactions (PCRs) that were to be subsequently used for molecular cloning were carried out using Bio-X-Act Long DNA Polymerase (Bioline) with 11.1 x PCR buffer. All other PCRs were performed using *Taq* DNA Polymerase (ABgene) and 11.1 x PCR buffer. The components of 11.1 x PCR buffer are 45 mM Tris-Cl (pH 8.8), 11 mM ammonium sulphate, 4.5 mM magnesium chloride, 6.7 mM β-mercaptoethanol, 4.4 μM EDTA (pH 8.0), 113 μg ml^{-1} BSA and 1 mM each of dATP, dCTP, dGTP and dTTP. Typical reactions contained 0.5 units μl^{-1} of polymerase, 1 x buffer and 1 pmol μl^{-1} of forward and reverse primers. Reactions contained 50-100 ng of template DNA in a total volume of 20-50 μl, made up with distilled deionised water. A typical reaction involved 35 cycles of a denaturation step at 95°C for 30 seconds, an annealing step at 50-70°C for 30 seconds and an extension step at 72°C for 1 minute per 1 kb of PCR product.

Oligonucleotide primers for construction of reporter plasmids, confirmation of plasmid integration into the genome of *C. albicans* and the construction of probes for northern blotting are shown in Tables 2.7, 2.8 and 2.9 respectively. Primers were purchased from Invitrogen, Sigma or the University of Leicester's Protein and Nucleic Acid Laboratory.

2.6 Site directed mutagenesis

The mutagenesis strategy was based on the QuikChange® XL Site Directed Mutagenesis kit from Stratagene. This is a PCR-based method that uses two complementary primers containing the desired mutations to amplify both strands of the plasmid. The parental plasmid DNA is then digested to completion and only the mutated plasmid DNA is transformed into *E. coli*. Mutagenesis primers are shown in Table 2.10 and were designed to be between 25 and 60 bases in length, to include the desired mutation in the middle of the primer and to have a melting temperature of 68°C or higher. Primers were purchased from Thermo Scientific and were purified by RP-HPLC (Reversed Phase-High Performance Liquid Chromatography). The mutant strands were synthesised in a 50 μl reaction comprising 10-50 ng of template plasmid, 0.5 units μl^{-1} of Bio-X-Act polymerase, 1 x PCR buffer, 1 pmol^{-1} each of forward and reverse primers and the remainder made up with distilled deionised water. The cycling

Table 2.6 Sequencing primers

Primer name	Sequence
LacZ seq F	GATCGCTGGTACTGAGCTCACGC
LacZ seq R	TGAGCGTCAACAGTATTAAC

Table 2.7 Primers used in the construction of reporter plasmids

Primer name	Sequence	Features
CaCTR1 776 F	GATCGTCGACGTCCCTACAGTAAACAAGGC	*Sal*I site
CaCTR1 387 F	GATCGTCGACCTCATCAATCTCTTACCAATCC	*Sal*I site
CaCTR1 263 F	GATCGTCGACCTTTGACCCTTTAAATACC	*Sal*I site
CaCTR1 202 F	GATCGTCGACCTTGAATTCAAACCCCATATTC	*Sal*I site
CaCTR1 776 R	GATCCCCGGGAATGTAAGGTTAAATGTGGTGTTG	*Xma*I site
CaFRE7 796 F	GATCCTCGAGGATTTCTTGCATTGTTTCGG	*Xho*I site
CaFRE7 796 R	GATCCCCGGGTTCAATGGTTTCAAGAAAATTTCAAG	*Xma*I site
CaMAC1 683 F	GATCCTCGAGCGGCATTGTTTAAGACTGAAATTGGG	*Xho*I site
CaMAC1 683 R	GATCCCCGGGTCCTTATTCAGTCTTGCTTTTTGGAGG	*Xma*I site

Table 2.8 Primers for confirmation of plasmid integration

Primer name	Sequence
RP10 F	CCAGCTCTCACAGATACTC
URA3 R	GGTGATGGATTAGGACAAC

Table 2.9 Primers for construction of northern blotting probes

Primer name	Sequence	Probe
ACT1 F	GGTAGACCAAGACATCAAGG	*CaACT1*
ACT1 R	GAACCACCAATCCAGACAGAG	*CaACT1*
AOX2 F	TCCAGCTTTCCATCAACCAA	*AOX2*
AOX2 R	GTGAACCAACTTGGTTTACCA	*AOX2*
CaCTR1 +60	GGCAATGTCGGCGAATTCAGC	*CaCTR1*
CaCTR1+568	CTCTAGAAATAGTTGATGCC	*CaCTR1*
FRE7 F	GCCTACATGAAGTACAAGC	*CaFRE7*
FRE7 R	GGACCACGTGAATGACTG	*CaFRE7*
OrfMAC1 F	CTAGCAGTAGAACGTTCGTG	*CaMAC1*
OrfMAC1 R	GCGTATCTCGATTGTTGACC	*CaMAC1*
SFU1 F	GTCCTGTAAATCTTAAACGG	*SFU1*
SFU1 R	GATGATTGCATTGGTGAATG	*SFU1*

Table 2.10 Mutagenesis primers

Primer name	Sequence	Features
CaCTR1-1 F	GGATTGGTAAGAGATTGATGAGCATATGCAAATTTTCAATTACC	*Nde*I site added
CaCTR1-1 R	GGTAATTGAAAATTTGCATATGCTCATCAATCTCTTACCAATCC	*Nde*I site added
CaCTR1-2 F	GGGTCAAAGAATATTTGTTAACACTTGAGATTTAATTATTAGGACTTAGTC	*Hpa*I site added
CaCTR1-2 R	GACTAAGTCCTAATAATTAAATCTCAAGTGTTAACAAATATTCTTTGACCC	*Hpa*I site added
CaCTR1-3 F	GGAAAGAAAGAAAGAAAAATACGCGTAAAATAAAAAGGTATTTAAAGGG	*Mlu*I site added
CaCTR1-3 R	CCCTTTCCCTACCTTTTTATTTTACGCGTATTTTTCTTTCTTTCTTTCC	*Mlu*I site added
CaFRE12-1 F	GTGTCTGCAAAATGGCCAAATTATTGGAACCCCG	*Msc*I site added
CaFRE12-1 R	CGGGGTTCCAATAATTTGGCCATTTTGCAGACAC	*Msc*I site added
CaFRE12-2 F	CCCCGAAACAGTAATAATTACAAAGTAATAAGCTTAAAATTTTCACATTATAATTACG	*Hind*III site added
CaFRE12-2 R	CGTAATTATAATGTGAAAATTTTAAGCTTATTACTTTGTAATTATTACTGTTTCGGGG	*Hind*III site added
CaMAC1-1 F	CCATTCCCAATCGGGAAATGATAAATTTGGCCATCCCGCTTC	*Hae*III site added
CaMAC1-1 R	GAAGCGGGATGGCCAAATTTATCATTTCCCGATTGGGAATGG	*Hae*III site added

reaction for mutagenesis involved one cycle at 95°C for one minute, 18 cycles of a denaturation step at 95°C for 50 seconds, an annealing step at 60 °C for 50 seconds and an extension step at 68°C for one minute per 1 kb of plasmid, with a final cycle at 68°C for 8 minutes. The Qiagen MinElute™ PCR purification kit was used to clean up the PCR reaction, following the manufacturer's instructions except an additional step at the end of the protocol was added to elute the DNA with another 10 μl of elution buffer (10 mM Tris-Cl, pH 8.5). The total elution from the Qiagen kit (20 μl) was digested overnight with *Dpn*I (New England Biolabs) to digest the parental *dam*-methylated plasmid DNA and prevent its transformation. Digestion with *Dpn*I did not affect the newly synthesised mutant plasmid DNA that was not methylated. The digest was then transformed into XL-10 Gold *E. coli* as described previously. During transformation the nicks in the newly synthesised mutant plasmid were repaired to recircularise the plasmid.

2.7 Southern and northern blotting and hybridisation

Southern blotting

DNA was transferred from agarose gels to nylon membrane using a method adapted from (Southern, 1975) and using an additional depurination step (Wahl *et al.*, 1979). After visualisation of DNA, the agarose gel was rinsed in distilled water and washed in depurinating solution (0.25 M hydrochloric acid) for 20 minutes with agitation. The gel was rinsed with distilled water and then washed in denaturing solution (0.5 M sodium hydroxide, 1 M sodium chloride) for 20 minutes with agitation. The gel was rinsed with distilled water and washed in neutralising solution (0.5 M Tris-Cl pH 7.4, 3 M sodium chloride for 20 minutes with agitation. The blotting apparatus comprised a plastic tray containing 20 x SSC with a glass plate resting on it. The glass plate was covered with 3 MM paper soaked in 20 x SSC with both edges of the paper in contact with the 20 x SSC to act as a wick. The gel was rinsed with distilled water and placed on top of the 3 MM paper. A nylon filter cut to the same size of the gel and two pieces of 3 MM paper were soaked in 6 x SSC and the filter was placed on top of the gel. This was followed by the 3 MM paper, a stack of paper towels and a glass plate. A weight was placed on top of the glass plate and transfer was performed overnight. When transfer was completed, the nylon filter was dried and the DNA fixed using 700,000 $\mu J\ cm^{-2}$ of energy in a UV crosslinker (Amersham Biosciences).

Northern blotting

RNA was transferred to nylon membrane by northern blotting immediately after denaturing agarose gel electrophoresis. No washing steps were required before northern blotting. The northern blotting apparatus was the same as for Southern transfer as described above, except that 10 x SSC was used instead of 20 x SSC and 3 x SSC was used instead of 6 x SSC. Transfer was performed overnight and the RNA was fixed using 700,000 μJ cm^{-2} of energy in a UV crosslinker (Amersham Biosciences).

Radioactive labelling of probes

Radioactive α-^{32}P d-CTP was used to label probes for Southern and Northern blotting using random hexamers as primers (Feinberg & Vogelstein, 1983). Probes were diluted to contain 30 ng of DNA in a total volume of 16 μl. The diluted probes were denatured by boiling for 5 minutes and chilling on ice for 5 minutes. The probes were labelled by adding 5 μl of OLB (Oligolabelling buffer), 1 μl of BSA, 1 μl Klenow fragment of DNA Pol I and 2.5 μl of α-^{32}P d-CTP. OLB was made from a mixture of solutions A, B and C in the ratio 2:5:3. Solution A contained 1 ml Solution O (1.25 M Tris-Cl, pH 8.0, 0.125 M magnesium chloride), 18 μl β-mercaptoethanol and 5 μl each of dATP, dTTP and dGTP at a concentration of 100 μM. Solution B contained 2 M HEPES (pH 6.6) and Solution C contained random hexamers (GE Healthcare) resuspended in TE (pH 8.0) at a concentration of 90 OD_{600} units ml^{-1}. Probes were labelled for at least one hour at 37°C. Unincorporated nucleotides were removed using illustra NICK™ columns (GE Healthcare). Columns were equilibriated with one column volume of elution buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1 % SDS). The total labelling reaction was applied to the column and eluted twice with 400 μl elution buffer. The second elution was denatured by boiling for 10 minutes and chilling on ice for 10 minutes and then used in hybridisation reactions.

Hybridisation of radiolabelled probes

Filters were pre-hybridised in tubes with constant rotation for at least one hour at 65°C in Church Gilbert's buffer (0.5 M Na_2HPO_4, 0.5 M NaH_2PO_4, 7 % SDS, 1 mM EDTA). Radiolabelled probe was added and hybridised to the filter overnight with

constant rotation at 65°C. The filter was then washed at 65°C with constant rotation with several changes of wash buffer (3 x SSC, 0.1 % SDS) until no further radioactive material was washed off the filters. Filters were then dried, wrapped in Saran wrap and placed into autoradiography cassettes with X-ray film. The X-ray films were exposed at -80°C and then developed.

2.8 β-galactosidase assays (Rupp, 2002)

A single colony was used to inoculate 10 ml of YPD and the culture was incubated for approximately six hours at 30°C with shaking. The culture was harvested by centrifugation at 4000 rpm for 5 minutes and the pellet was washed twice in sterile distilled water and resuspended in a final volume of 1 ml of sterile distilled water. The optical density of the suspension at 600 nm was determined to estimate the cell density based on previous growth curve experiments. A culture containing approximately $3x10^4$ cells ml^{-1} in 5 ml of MD containing high copper and high iron was grown overnight at 30°C with shaking. The next morning the cultures were harvested, washed and resuspended in a final volume of 1 ml sterile distilled water as previously. The same overnight culture was used to inoculate 5 ml of MD with different supplements to a cell density of $2x10^6$ cells ml^{-1}. This culture was grown at 30°C with shaking for 5 hours until it reached exponential growth and a concentration of approximately 1 x 10^7 cells ml^{-1} and the culture was then harvested by centrifugation at 4000 rpm for 5 minutes. The cells were washed twice in 1 ml of sterile water and the pellets resuspended in 1 ml of ice cold Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM potassium chloride, 0.1 mM magnesium chloride, 50 mM β-mercaptoethanol). 200 μl was removed and used to measure the OD_{600} of the culture. The remaining 800 μl was used to carry out the assay and the cells were permeabilised by adding 60 μl chloroform and 40 μl 0.1 % SDS and vortexing for 10 seconds. A sample containing 800 μl Z buffer only was also included in the reaction as a negative control. The samples were preincubated at 30°C for approximately 5 minutes. The reaction was started by adding 200 μl of ONPG (4 mg ml-1 in Z buffer), vortexing briefly to mix, and the time was recorded. The samples were incubated at 30°C until a yellow colour could be seen in the sample tubes. The reaction was stopped by adding 400 μl of 1 M sodium carbonate and the time was recorded to calculate the reaction time. Samples were centrifuged at 13000

rpm for 5 minutes and the absorbance of 1 ml of the supernatants was measured at OD_{420} and OD_{550}. The negative control sample containing Z buffer only was used as a blank. Absorbance at OD_{420} measures the yellow colour produced when ONPG is degraded and absorbance at OD_{550} is the light scattering by cell debris. Units of β-galactosidase activity was calculated and corrected for light scattering due to cell debris using the following formula.

Activity = OD_{420} – (reaction volume x OD_{550}) / (OD_{600} x culture volume x time)

The reaction volume is 1.5 ml total volume of the reaction and the culture volume is 0.8 ml of the original culture used in the assay.

Chapter 3

Analysis of putative CaMac1p binding sites in the *CaCTR1* promoter

3.1 Introduction

Previous work in our laboratory identified a *C. albicans* high affinity copper transporter gene by screening a *C. albicans* genomic library for clones that could rescue a *S. cerevisiae* mutant that lacks the high affinity copper transporter genes *CTR1* and *CTR3* (Knight *et al.*, 1996; Marvin *et al.*, 2003). This approach identified a gene with 39.6 % similarity to *S. cerevisiae CTR1*, named *CaCTR1*, that could rescue all phenotypes associated with defects in copper transport, including slow growth in low copper medium, sensitivity to oxidative stress and the inability to utilise non-fermentable carbon sources (Dancis *et al.*, 1994a; Dancis *et al.*, 1994b; Knight *et al.*, 1996; Marvin *et al.*, 2003). A homozygous *C. albicans ctr1Δ/ctr1Δ* strain showed the same phenotypes associated with defects in copper transport as the *S. cerevisiae ctr1Δctr3Δ* mutant (Marvin *et al.*, 2003). The *Cactr1Δ/Cactr1Δ* mutant also showed an increase in pseudohyphal growth in low copper conditions and a 96 % decrease in high affinity iron uptake (Marvin *et al.*, 2003; Marvin *et al.*, 2004).

We also showed that *CaCTR1* transcript levels increased in low copper conditions when *CaCTR1* was expressed in both *C. albicans* and *S. cerevisiae* (Marvin *et al.*, 2003). Heterologous expression of *CaCTR1* in *S. cerevisiae* requires the transcriptional activator gene *ScMAC1* for activation in low copper conditions. There are three sequences present in the *CaCTR1* promoter that are identical to the ScMac1p consensus binding site (Martins *et al.*, 1998; Marvin *et al.*, 2003). This evidence suggested that *CaCTR1* could be regulated by a similar *MAC1*-like activator in *Candida albicans* and we identified a potential regulator in the *C. albicans* genome by homology to *ScMAC1* (Marvin *et al.*, 2004). The predicted protein for *CaMAC1* shares 35.0 % similarity with ScMac1p, and both proteins contain an N-terminal copper fist DNA binding motif and two cysteine-rich copper sensing motifs in the C-terminal activation domain of the protein (Jensen & Winge, 1998; Jungmann *et al.*, 1993). A homozygous *C. albicans mac1Δ/mac1Δ* mutant also shows the same

phenotypes associated with defective copper transport as a *S. cerevisiae mac1*Δ strain such as slow growth in low copper medium, inability to utilise non-fermentable carbon sources and sensitivity to oxidative stress (Jungmann *et al.*, 1993; Marvin *et al.*, 2004). Northern blots showed that the *C. albicans MAC1* homologue (*CaMAC1*) was required for activation of *CaCTR1* in low copper conditions (Marvin *et al.*, 2004). The *CaMAC1* gene appears to be similar to its *S. cerevisiae* homologue *ScMAC1,* and CaMac1p may also function in transcriptional regulation in response to copper levels.

The *S. cerevisiae CTR1* promoter contains two repeats of the consensus sequence TTTGC(T/G)C(A/G) and at least two repeats of this sequence are also present in the promoters of the copper-regulated genes *CTR3, FRE1* and *FRE7* (Labbe *et al.*, 1997; Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997). Electrophoretic mobility shift assays have demonstrated that purified Mac1p can bind directly to this sequence (Yamaguchi-Iwai *et al.*, 1997), and dimethyl sulphate footprinting of the *CTR3* promoter shows that the same sequences in this promoter are occupied in low copper conditions (Labbe *et al.*, 1997). The consensus sequence was named the copper responsive element (CuRE) and extensive point mutagenesis of CuREs in the promoters of *CTR1, CTR3, FRE1* and *FRE7* has shown that deviation from the consensus results in a reduction of reporter gene activation and Mac1p binding (Jensen *et al.*, 1998; Labbe *et al.*, 1997; Martins *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1997).

Further analysis of the *CTR1* promoter showed that both CuREs were required for copper-responsive transcription, and that mutation of the CuRE sequences significantly reduced the binding of Mac1p (Jensen *et al.*, 1998). Mutation of the two CuREs in the *FRE1* promoter also abolishes copper-regulated transcription (Labbe *et al.*, 1997). One CuRE in the *FRE7* promoter was sufficient for copper-responsive transcription, but these levels of transcription were significantly reduced compared to a wild type *FRE7* promoter containing three CuREs (Martins *et al.*, 1998). A truncated *CTR3* promoter containing just one CuRE was able to activate *lacZ* transcription two-fold in low copper conditions, but this was also significantly reduced compared to eight-fold activation of *lacZ* by a full-length *CTR3* promoter (Labbe *et al.*, 1997). Activation by a truncated *CTR3* promoter of 248 bp containing

two CuREs was also much lower than activation by the wild type promoter (Labbe *et al.*, 1997). At least two CuREs are therefore required for wild-type expression levels of *CTR1*, *CTR3* and *FRE7* and this evidence also suggests that two CuREs are likely to be essential for copper-dependent expression of *FRE1*.

Three sequences that match the ScMac1p copper response element (CuRE) have been identified in the *CaCTR1* promoter. An additional sequence is also present in the *CaCTR1* promoter that contains the core sequence of GCTCA, but not the preceding TTT sequence. In electrophoretic mobility shift assays, purified ScMac1p binding domain (residues 1-159) does not bind to CuREs containing mutations of the three thymidine residues at the 5'end of the CuRE (Jensen *et al.*, 1998). The full CuRE sequence is therefore also likely to be required for binding of CaMac1p and the transactivation activity of CaMac1p bound to the promoter. The work described in this chapter uses a β-galactosidase reporter strategy to analyse the promoter activity of wild-type and mutant *CaCTR1* promoters to determine if the three putative CuREs have a function in the transcriptional regulation of *CaCTR1*.

Using reporter genes in *Candida albicans*

The expression of heterologous genes, including reporter genes, in *C. albicans* has been restricted due to the non-conventional decoding of the CTG codon as serine rather than leucine (Santos & Tuite, 1995; White *et al.*, 1995). As a result, genes containing CTG codons are transcribed, but the proteins cannot be detected by Western blot analysis indicating that the proteins cannot be synthesised correctly (Cormack *et al.*, 1997; Morschhauser *et al.*, 1998). The widely-used *E. coli* β-galactosidase reporter gene *lacZ* was not expressed in *C. albicans* due to the high number of 51 CTG codons in this gene (Kalnins *et al.*, 1983; Leuker *et al.*, 1992). The first reporter gene developed for use in *C. albicans* was the β-galactosidase gene *LAC4* from the yeast *Kluyveromyces lactis* (Leuker *et al.*, 1992). The *K. lactis LAC4* reporter gene was subsequently used to show that transcription controlled by the alternative oxidase (*AOX2*) promoter increases in response to stress conditions including cyanide, glycerol and oxidants (Huh & Kang, 2001). Uhl & Johnson (2001) investigated the effect on reporter activity of the two CTG codons in *K. lactis LAC4*. Point mutagenesis was used to change the two CTG codons to the *C. albicans* leucine

codon TTA and a single copy was integrated into the genome (Uhl & Johnson, 2001). Wild-type and mutant *LAC4* genes all showed very low levels of β-galactosidase activity when fused to the *MAL2* promoter (Uhl & Johnson, 2001). The levels of β-galactosidase activity of *LAC4* fusions that were observed by Uhl & Johnson were very low compared to the β-galactosidase levels detected by Leuker *et al.* This is likely to be because the reporter plasmid constructed by Leuker *et al.* was present in higher numbers than the single integrated plasmids in the study by Uhl & Johnson. The higher copy number of plasmids would increase β-galactosidase activity but this also causes variance between constructs and it is difficult to compare the results of different experiments.

Uhl & Johnson also identified the *lacZ* gene from *Streptococcus thermophilus* as a potential reporter gene in *Candida albicans.* The *S. thermophilus lacZ* gene has just one CTG codon, which is also in a region of the gene that is not conserved between β-galactosidases and is not likely to be essential for the function of the protein (Schroeder *et al.*, 1991; Uhl & Johnson, 2001). *S. thermophilus lacZ* was used to construct reporter plasmids, with *lacZ* fused to a variety of promoters, that would integrate into the *C. albicans* genome at a single locus. Quantitative β-galactosidase assays demonstrated that *lacZ* could be expressed constitutively (*ACT1-lacZ* fusion), induced by maltose (*MAL2-lacZ* fusion) and induced by hyphal growth (*HWP1-lacZ* fusion). However, mutagenesis to alter the CTG codon did not increase the production of β-galactosidase and the wild-type *S. thermophilus lacZ* gene could be used (Uhl & Johnson, 2001). This reporter system has since been used by several other workers to analyse regulation of multi-drug resistance genes (Rognon *et al.*, 2006), the transcriptional repressor gene *RIM101* (Baek *et al.*, 2006) and the histidine kinase gene *CHK1* (Li *et al.*, 2004).

For this study, a β-galactosidase reporter system using *Streptococcus thermophilus lacZ* was chosen because of the reproducibility, simplicity and cost effectiveness of this method. The reporter plasmid plac-poly used in this study was adapted at the University of Aberdeen from the CIp10 integrating plasmid (Murad *et al.*, 2000). Integrating plasmids are maintained more stably than episomal plasmids in *C. albicans* and do not show variance in copy number. Plasmids with a variable copy

number can alter the phenotype of the host strain containing episomal plasmids (De Backer *et al.*, 2000). CIp10 contains an ampicillin resistance gene (*bla*) for selection in *E. coli* and *CaURA3* for selection in *C. albicans*. The plasmid contains a copy of the ribosomal protein gene *CaRPS10* with unique *Stu*I restriction sites added, and following digestion with *Stu*I the plasmid integrates into the *C. albicans* genome by homologous recombination at the *CaRPS10* locus, as illustrated in Figure 3.1. *CaRPS10* is highly expressed during exponential growth and Southern blot data suggests that this gene is present at two separate loci in the genome (Swoboda *et al.*, 1995). This locus can therefore be disrupted without any effect on growth and any genes integrated at this location are highly expressed (Murad *et al.*, 2000). Transformation with CIp10 is also very efficient compared to transformation with other plasmids (Care *et al.*, 1999; Murad *et al.*, 2000). The reporter plasmid plac-poly (see Figure 2.1) has the *Streptococcus thermophilus lacZ* gene inserted into CIp10 between the *bla* and *CaRPS10* genes in the same orientation, and the multiple cloning site is located immediately upstream of the translation start of *lacZ* (Brown *et al.*, personal communication).

3.2 Construction and analysis of a *CaCTR1* promoter-*lacZ* fusion plasmid

The *CaCTR1* promoter from -776bp to -3bp (relative to the ATG) was amplified from *C. albicans* genomic DNA using primers *CaCTR1 776 F* and *CaCTR1 776 R* (Table 2.7), which incorporated restriction sites for *Sal*I and *Xma*I respectively. The PCR product and plac-poly were digested with *Sal*I and *Xma*I and the fusion plasmid pAWC1 was constructed by ligating the *CaCTR1* promoter to the MCS of plac-poly. Plasmids pAWC1 and plac-poly were linearised by digestion with *Stu*I and transformed into *C. albicans* strain BWP17 (wild type). *CaURA3* was used as the selectable marker to identify transformants in the parental strain BWP17 (*ura3*Δ/*ura3*Δ). Plac-poly that did not contain a promoter to activate *lacZ* expression was included as a negative control to determine the background level of β-galactosidase activity.

To investigate the role of *CaMAC1* in the regulation of *CaCTR1*, transformation of the fusion plasmid into a *mac1*Δ/*mac1*Δ strain (MEM-m2) was required. However,

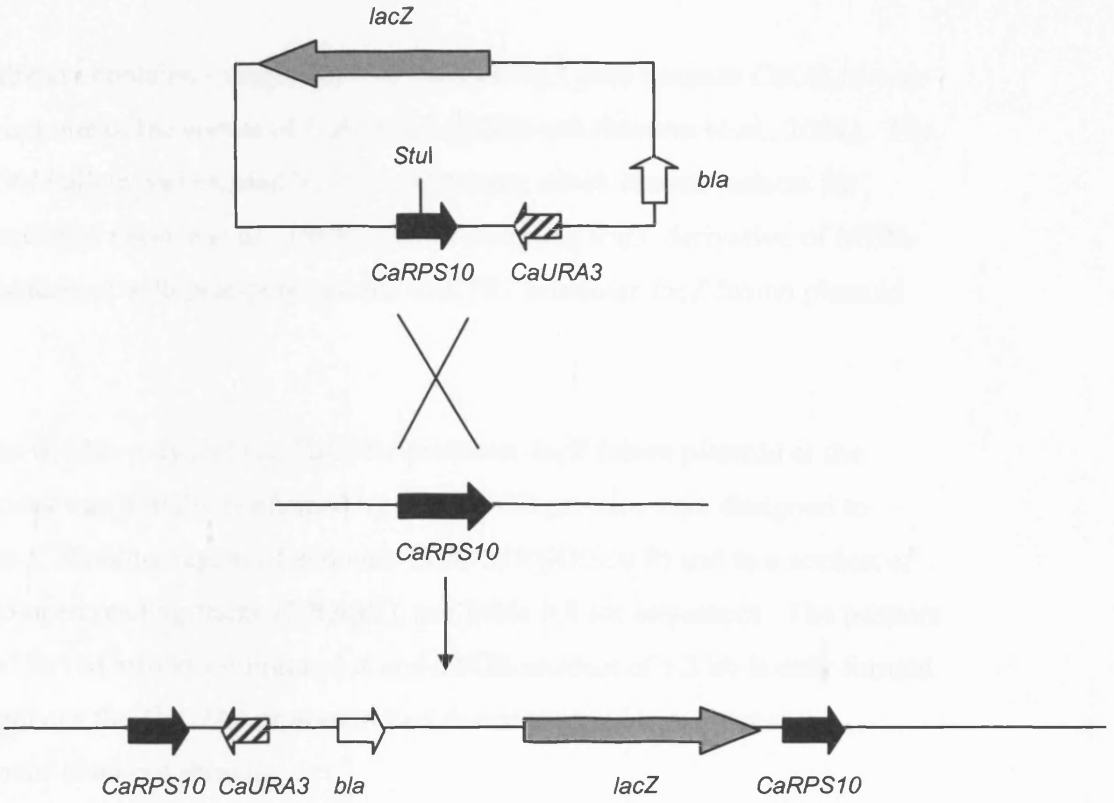


Figure 3.1 Integration of plac-poly into the *C. albicans* genome at the *CaRPS10* locus
The copy of *CaRPS10* in plac-poly contains artificially inserted *Stu*I restriction sites. Following digestion with *Stu*I the two halves of *CaRPS10* homologously recombine with the genomic copy of *CaRPS10* and the plasmid is integrated into the genome at the *CaRPS10* locus.

MEM-m2 already contains a single copy of the *CaURA3* gene because *CaURA3* was used to disrupt one of the copies of *CaMAC1* in MEM-m2 (Marvin *et al.*, 2004). The single *CaURA3* allele was excised by FOA treatment, which counter-selects for uridine prototrophy (Boeke *et al.*, 1984), and the resulting *ura3*$^-$ derivative of MEM-m2 was transformed with plac-poly and the *CaCTR1* promoter-*lacZ* fusion plasmid pAWC1.

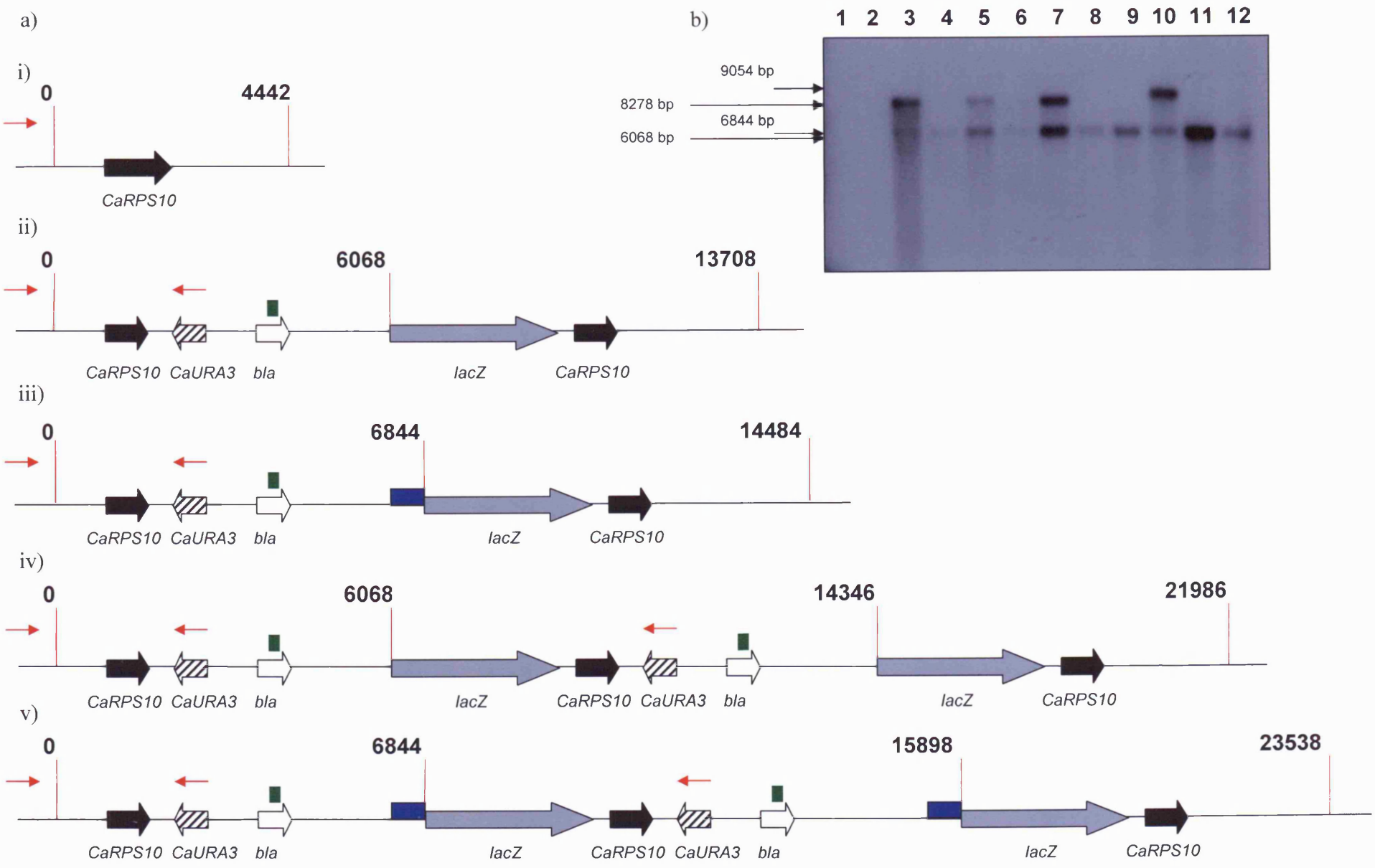
The presence of plac-poly and the *CaCTR1* promoter-*lacZ* fusion plasmid at the *CaRPS10* locus was initially confirmed by PCR. PCR primers were designed to anneal to the 5' flanking region of genomic *CaRPS10* (RPS10 F) and to a section of the *CaURA3* open reading frame (URA3R), see Table 2.8 for sequences. The primers are indicated by red arrows on Figure 3.2 and a PCR product of 1.3 kb is only formed when plac-poly or the *CaCTR1* promoter-*lacZ* fusion plasmid is present at the *CaRPS10* locus (data not shown).

Southern blot analysis was used to confirm integration of the plasmids at the *CaRPS10* locus and to determine the number of plasmid copies integrated into the genome. Using transformants containing one copy of the plasmid ensures that only one copy of *lacZ* is being analysed in each strain and that any differences of β-galactosidase activity are due to the *CaCTR1* promoter cloned into the plasmids. Genomic DNA from transformants was digested with *Acc*I, resolved on a 1 % agarose gel and Southern blotted as described in Chapter 2. To generate a probe for the *bla* gene, plac-poly was digested with *Acl*I to give two products of 373 bp and 7905 bp. The 373 bp fragment of the *bla* gene was purified using the Qiagen MinElute™ Gel Extraction kit and used a probe for Southern blotting. The blot was hybridised with a α^{32}P-labelled *bla* probe and exposed to X-ray film. The presence of one band of either 6844 bp or 6068 bp on the Southern blot indicates that one copy of the plasmid integrated at *CaRPS10* and at least one of each type of transformant contains a single copy of the plasmid (Figure 3.2). The BWP17 (wild type) and *mac1*Δ/*mac1*Δ strains with one copy of plac-poly were named AWP1 (Lane 4) and AWP1.1 (Lane 8) respectively and the wild type and *mac1*Δ/*mac1*Δ strains with one copy of the *CaCTR1* promoter-*lacZ* fusion plasmid were named AWC1 (Lane 9) and AWC1.1 (Lane 11) respectively.

Figure 3.2 Southern analysis of plasmid integration

a) The site of integration of *lacZ*-fusion plasmids at the *RPS10* locus is shown with *Acc*I restriction sites as vertical red lines. The locations where primers RPS10F and URA3R bind are shown as red arrows, the location of the *bla* probe for Southern blotting is shown in green and the *CaCTR1* promoter sequence is shown in blue. Five possible results of transformation with plac-poly or *CaCTR1* promoter-*lacZ* fusion plasmid are shown (i-v).

b) Genomic DNA was digested with *Acc*I, resolved on a 1 % agarose gel and Southern blotted as described in Chapter 2. The blot was then hybridised with α ^{32}P-labelled fragment of *bla* and exposed to X-ray film. Lane 1: BWP17 (wild type) with no plasmid shows no fragments containing *bla*, as illustrated in a) (i). Lane 2: *mac1*Δ/*mac1*Δ with no plasmid shows no fragments containing *bla* (i). Lane 3: BWP17 (wild type) transformed with plac-poly shows two fragments containing *bla* of 6068 bp and 8278 bp as illustrated in (iv). Lane 4: BWP17 (wild type) transformed with plac-poly shows one fragment of 6068 bp (ii) and is named AWP1. Lane 5: BWP17 (wild type) transformed with plac-poly shows two fragments of 6068 bp and 8278 bp (iv). Lane 6: *mac1*Δ/*mac1*Δ transformed with plac-poly shows two fragments of 6068 bp and 8278 bp (iv). Lane 7: *mac1*Δ/*mac1*Δ transformed with plac-poly shows two fragments of 6068 bp and 8274 bp (iv). Lane 8: *mac1*Δ/*mac1*Δ transformed with plac-poly shows one fragment of 6068 bp (ii) and is named AWP1.1. Lane 9: BWP17 (wild type) transformed with *CaCTR1* promoter-*lacZ* fusion (plasmid pAWC1) shows one fragment of 6844 bp (iii) and is named AWC1. Lane 10: *mac1*Δ/*mac1*Δ transformed with *CaCTR1* promoter-*lacZ* fusion (plasmid pAWC1) shows two fragments of 6844 bp and 9054 bp (v). Lane 11: *mac1*Δ/*mac1*Δ transformed with *CaCTR1* promoter-*lacZ* fusion (plasmid pAWC1) shows one fragment of 6844 bp (iii) and is named AWC1.1. Lane 12: *mac1*Δ/*mac1*Δ transformed with *CaCTR1* promoter-*lacZ* fusion (plasmid pAWC1) shows one fragment of 6844 bp (iii).


a)
i)
0
4442
CaRPS10
ii)
0
6068
13708
CaRPS10 CaURA3 bla lacZ CaRPS10
iii)
0
6844
14484
CaRPS10 CaURA3 bla lacZ CaRPS10
iv)
0
6068
14346
21986
CaRPS10 CaURA3 bla lacZ CaRPS10 CaURA3 bla lacZ CaRPS10
v)
0
6844
15898
23538
CaRPS10 CaURA3 bla lacZ CaRPS10 CaURA3 bla lacZ CaRPS10
b)
1 2 3 4 5 6 7 8 9 10 11 12
9054 bp
8278 bp
6844 bp
6068 bp

Analysis of the β-galactosidase activity of the wild-type *CaCTR1* promoter-*lacZ* fusion

Strains containing one copy of the fusion plasmid were tested for β-galactosidase activity during exponential phase after 5 hours of growth as described in Materials and Methods 2.8. Transformants containing the fusion plasmid pAWC1 were grown in high and low copper media and β-galactosidase assays were carried out. BWP17 (wild type) containing the fusion plasmid showed a three fold increase in β-galactosidase activity in low copper conditions (Figure 3.3). When the *CaCTR1* promoter-*lacZ* fusion plasmid is transformed into MEM-m2 (*mac1*Δ/*mac1*Δ) the β-galactosidase activity is decreased by 70 % - 90 % compared to activity in the wild type (Figure 3.3). There is also no difference in β-galactosidase activity in different copper concentrations in *mac1*Δ/*mac1*Δ (Table 3.1), showing that *CaMAC1* is needed for copper-responsive activation of *lacZ* by the *CaCTR1* promoter. The activation of the *CaCTR1* promoter in low copper is consistent with northern blot data that shows an increase in *CaCTR1* transcripts in low copper and that *CaMAC1* is required for *CaCTR1* transcription (Figure 3.4). Cultures were grown under the same conditions and to the same stage of growth for both RNA extraction and β-galactosidase assays.

As a control, the background β-galactosidase activity of plac-poly was determined from transformants containing plac-poly without a promoter to activate *lacZ* expression. The maximum β-galactosidase activity of the plac-poly control plasmids was over ten times lower than the activity of plasmids containing the *CaCTR1* promoter and was not affected by copper concentration (Figure 3.3, Table 3.1). This shows that the background β-galactosidase activity of plac-poly is very low and β-galactosidase activity of fusion plasmids should accurately represent the effects of any promoter fused to *lacZ*.

3.3 Mutagenesis of the *CaCTR1* promoter-*lacZ* fusion

A mutagenesis method based on the Stratagene XL-Quikchange ® Site-directed Mutagenesis Kit was used to introduce base substitutions in the putative CuREs within the existing *CaCTR1* promoter-*lacZ* fusion plasmid. The site directed mutagenesis method is described in Materials and Methods section 2.6 and used complementary primers (Table 2.10) incorporating the desired mutations to amplify

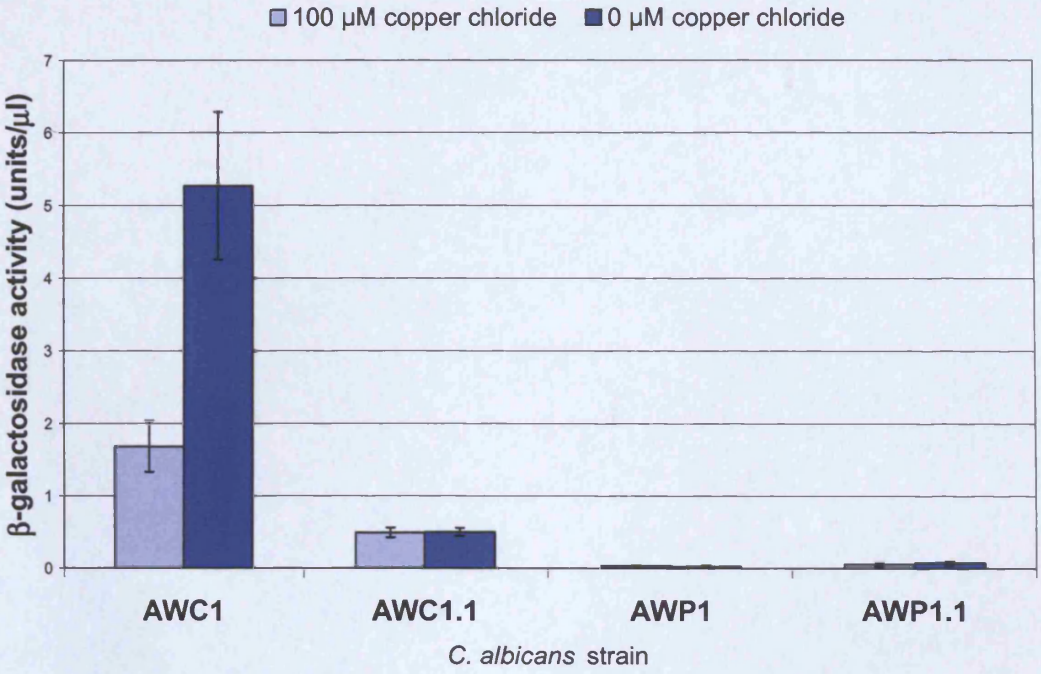


Figure 3.3 β-galactosidase activity of *CaCTR1-lacZ* fusions

BWP17 (wild type) containing *CaCTR1* promoter-*lacZ* fusion plasmid (AWC1), MEM-m2 (*mac*1Δ/*mac1*Δ) containing fusion plasmid (AWC1.1), BWP17 containing plac-poly only (AWP1) and MEM-m2 containing plac-poly only (AWP1.1) were tested for β-galactosidase activity. Cultures were grown in high and low copper media and β-galactosidase assays were carried out as described in Materials and Methods 2.8. This figure shows the mean results of three separate experiments with error bars of one standard deviation.

Table 3.1 Summary of β-galactosidase assays

The results of all β-galactosidase assays carried out in this chapter are summarised in this table, showing the statistical significance of each construct relative to the activity of the wild-type *CaCTR1* promoter-*lacZ* fusion construct AWC1. The results of a one-tailed student's t-test are shown as P values ($\alpha = 0.05$). The relative increase in activity in low copper is also shown with the statistical significance of this increase. Results are deemed to be statistically significant if the P value is less than 0.05.

			High copper		**Low copper**			
Strain	**Parental strain**	***CaCTR1* promoter mutations**	**B-galactosidase activity (units μl^{-1})**	**Difference from wild type (P value)**	**B-galactosidase activity (units μl^{-1})**	**Difference from wild type (P value)**	**Fold increase in low copper**	**Difference between copper levels (P value)**
AWC1	BWP17 (*MAC1*)	Wild-type	1.683	n/a	5.270	n/a	3.131	0.008
AWC1.1	*mac1Δ/mac1Δ*	Wild-type	0.495	0.018	0.502	0.007	1.014	0.456
AWP1	BWP17 (*MAC1*)	No promoter	0.036	0.007	0.027	0.006	0.750	0.095
AWP1.1	*mac1Δ/mac1Δ*	No promoter	0.058	0.007	0.076	0.006	1.310	0.191
AWC2	BWP17 (*MAC1*)	CuRE 1	0.486	0.011	1.645	0.011	3.385	0.001
AWC3	BWP17 (*MAC1*)	CuRE 2	1.262	0.194	7.783	0.038	6.167	0.002
AWC4	BWP17 (*MAC1*)	CuRE 3	2.416	0.111	4.644	0.255	1.922	0.018
AWC5	BWP17 (*MAC1*)	CuREs 1 & 2	0.533	0.041	0.797	0.011	1.495	0.210
AWC6	BWP17 (*MAC1*)	CuREs 1 & 3	1.289	0.102	2.124	0.017	1.648	0.000
AWC7	BWP17 (*MAC1*)	CuREs 2 & 3	2.349	0.024	10.925	0.002	4.651	0.001
AWC8	BWP17 (*MAC1*)	CuREs 1, 2 & 3	0.576	0.024	0.779	0.004	1.352	0.069

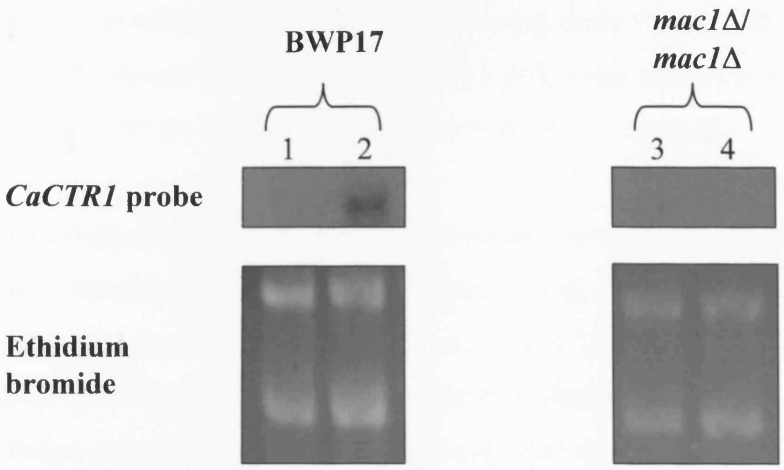


Figure 3.4 Northern blot analysis of *CaCTR1* transcripts

RNA was extracted from exponentially growing cultures of BWP17 (wild type, lanes 1-2) and MEM-m2 (*mac1*Δ/*mac1*Δ, lanes 3-4) as described in Chapter 2.3. Cultures were grown in MD media as described in Chapter 2.2 with high copper (lanes 1 & 3) or low copper (lanes 2 & 4). Denaturing formaldehyde RNA gels were northern blotted and hybridised with an α-^{32}P labelled fragment of *CaCTR1*. Total RNA stained with ethidium bromide is included as a loading control.

both strands of the existing *CaCTR1* promoter-*lacZ* fusion plasmid. The PCR reaction was then cleaned up using the Qiagen MinElute™ PCR purification kit and the original template plasmid was digested using *Dpn*I to target the parental *dam*-methylated plasmid DNA. Only the newly synthesised mutant plasmids were then transformed into the high-efficiency *E. coli* strain XL-10 Gold.

Different mutations of either two or three bases were made in each of the three CuREs and each mutation introduced a new restriction site. For example, CuRE1 (positioned at -375 bp relative to the ATG) was mutated from TTTGCTCA to TTTGCATA. This mutation also introduced an additional *Nde*I restriction site in the *CaCTR1* promoter-*lacZ* fusion plasmid, and *Nde*I digestion was used to screen for mutant plasmids. Each CuRE was mutated individually and in all possible combinations to generate seven mutant *CaCTR1* promoter-*LacZ* fusions. Figure 3.5 shows the sequence of all the mutant fusion plasmids and the additional restriction sites, along with the template plasmids and the primers used to construct the mutants. The mutated plasmids were confirmed by DNA sequencing and transformed into *C. albicans* BWP17 (wild type).

Integration of plasmids into the genome at *CaRPS10* was confirmed by PCR with primers RPS10F and URA3R, shown as red arrows in Figure 3.6, to give a product of 1.3 kb if the plasmid has integrated (data not shown). Genomic DNA from transformants was also digested with *Acc*I, resolved on a 1 % agarose gel and Southern blotted as described in Chapter 2. The blot was then hybridised with an α ^{32}P-labelled fragment of *bla* and exposed to X-ray film. BWP17 (wild type) was transformed with fusion plasmids pAWC2-8 containing different mutations of the CuREs in the *CaCTR1* promoter (see Figure 3.5), and there is one band of 6844 bp present in Lanes 2-8 of Figure 3.6, indicating that all of the transformants contain one copy of a *CaCTR1* promoter-*lacZ* fusion plasmid integrated at *CaRPS10* as illustrated in Figure 3.6 a (ii). The *C. albicans* strains in Lanes 2-8 were named AWC2-8 respectively and used for further analysis. Lane 1 contains wild type DNA without any plasmids as a negative control and no bands were detected in this lane.

Figure 3.5 Sequences of mutant binding sites in the *CaCTR1* promoter

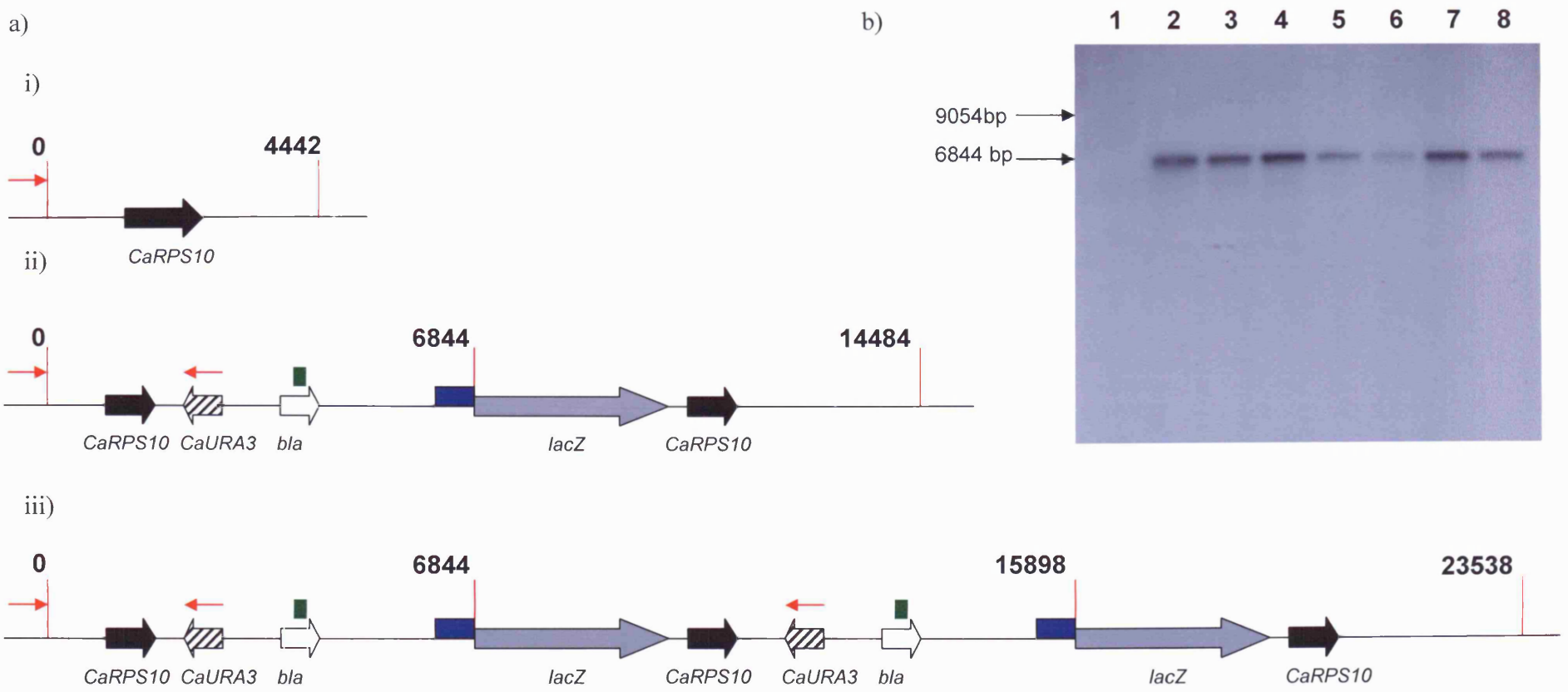
The figure below shows the positions of the copper response elements (CuREs) in the *CaCTR1* promoter, relative to the ATG. CuRE sequences are in capitals, surrounding sequences in lower-case letters and all mutant bases in red. The table shows how mutant CuREs were created by PCR-based site-directed mutagenesis. The template plasmid was amplified using primers (see Table 2.10 for sequences) containing the desired mutation and an additional restriction site (shown in the last column). The parental DNA was digested using *Dpn*I and the mutant plasmid transformed into *E. coli*. Mutants were screened by digestion with the additional restriction sites and confirmed by sequencing. The plasmids containing single CuRE mutations were used as templates to create the double and triple mutations indicated in the table.

Plasmid	Template	Sequence at binding sites			Primers	Restriction sites added
		CuRE 1	CuRE 2	CuRE 3		
pAWC1	Genomic	aTTTGCTCAtt	aTGAGCAAAt	tTTTGCTCAt	None	None
pAWC2	pAWC1	aTTTGCATAtg	aTGAGCAAAt	tTTTGCTCAt	CaCTR1-1 F CaCTR1-1 R	*Nde*I (CA^TATG)
pAWC3	pAWC1	aTTTGCTCAtt	gTTAACAAAt	tTTTGCTCAt	CaCTR1-2 F CaCTR1-2 R	*Hpa*I (GTT^AAC)
pAWC4	pAWC1	aTTTGCTCAtt	aTGAGCAAAt	tTTTACGCGt	CaCTR1-3 F CaCTR1-3 R	*Mlu*I (A^CGCGT)
pAWC5	pAWC2	aTTTGCATAtg	gTTAACAAAt	tTTTGCTCAt	CaCTR1-2 F CaCTR1-2 R	*Hpa*I, *Nde*I
pAWC6	pAWC1	aTTTGCATAtg	aTGAGCAAAt	tTTTACGCGt	CaCTR1-3 F CaCTR1-3 R	*Mlu*I, *Nde*I
pAWC7	pAWC4	aTTTGCTCAtt	gTTAACAAAt	tTTTACGCGt	CaCTR1-2 F CaCTR1-2 R	*Hpa*I, *Mlu*I
pAWC8	pAWC6	aTTTGCATAtg	gTTAACAAAt	tTTTACGCGt	CaCTR1-2 F CaCTR1-2 R	*Mlu*I, *Hpa*I, *Nde*I

Figure 3.6 Southern blot analysis of *CaCTR1* promoter-*lacZ* fusion plasmid integrations

a) The site of integration of *lacZ*-fusion plasmids at the *RPS10* locus is shown with *Acc*I restriction sites as vertical red lines. The locations where primers RPS10F and URA3R bind are shown as red arrows, the location of the *bla* probe for Southern blotting is shown in green and the *CaCTR1* promoter sequence is shown in blue. Three possible results of transformation with *CaCTR1* promoter-*lacZ* fusion plasmids are shown (i-iii).

b) Genomic DNA was digested with *Acc*I, resolved on a 1% agarose gel and Southern blotted as described in Chapter 2. The blot was then hybridised with α ^{32}P-labelled fragment of *bla* and exposed to X-ray film. Lane 1: BWP17 (wild type) with no plasmid shows no fragments containing *bla*, as illustrated in a (i). Lanes 2-8: BWP17 (wild type) transformed with the following *CaCTR1* promoter-*lacZ* fusion plasmids. Lane 2: pAWC2 containing mutations in CuRE 1. Lane 3: pAWC3 containing mutations in CuRE 2. Lane 4: pAWC4 containing mutations in CuRE 3. Lane 5: pAWC5 containing mutations in CuRE 1 & CuRE 2. Lane 6: pAWC6 containing mutations in CuRE 1 & CuRE 3. Lane 7: pAWC7 containing mutations in CuRE 2 & CuRE 3. Lane 8: pAWC8 containing mutations in CuRE 1, CuRE 2 & CuRE 3. Lanes 2-8 all show one band at 6844 bp indicating that one copy of the plasmid has integrated at *CaRPS10*, as illustrated in Figure 3.4 a (ii). Two copies of the plasmid, as illustrated in a (iii), were not detected in any of the transformants. Transformants in lanes 2-8 were named AWC2-8 respectively and used for further analysis.


a)
i)
0
4442
CaRPS10
ii)
0
6844
14484
CaRPS10
CaURA3
bla
lacZ
CaRPS10
iii)
0
6844
15898
23538
CaRPS10
CaURA3
bla
lacZ
CaRPS10
CaURA3
bla
lacZ
CaRPS10
b)
1
2
3
4
5
6
7
8
9054bp
6844 bp

Analysis of the function of mutant *CaCTR1* promoter-*lacZ* fusions

The effect of mutating the CuREs on the ability of the *CaCTR1* promoter to activate the *lacZ* gene were tested. Wild type *C. albicans* (BWP17) containing fusion plasmids with wild type (pAWC1) or mutant *CaCTR1* promoters (pAWC2, pAWC3, pAWC4) were grown in high and low copper media and β-galactosidase assays were carried out as described in Materials and Methods 2.8. Mutation of CuRE 1 from TTTGCTCA to TTTGCATA caused a decrease in β-galactosidase activity of ~70 % in both high and low copper conditions compared to a wild-type *CaCTR1* promoter (Figure 3.7). Promoter activity therefore remains copper-regulated with a 3.4 fold increase in activation in low copper (Table 3.1).

Mutation of CuRE 2 does not significantly affect β-galactosidase activity in high copper (Table 1). In contrast, mutation of CuRE 2 resulted in a 40 % increase in β-galactosidase activity in low copper compared to a wild-type promoter (Figure 3.7). This results in an overall 6.2-fold increase in activation in response to low copper conditions in the CuRE 2 mutant (Table 3.1). The mutation of CuRE 2 would be expected to decrease β-galactosidase activity in a manner similar to the mutation of CuRE 1. Mutation of CuRE 3 does not cause any statistically significant effects on β-galactosidase activity (Figure 3.7, Table 3.1).

The contributions of the individual CuREs to activation of *lacZ* becomes more apparent when multiple CuREs are mutated. Mutation of CuREs 1 & 2 reduces β-galactosidase activity by 70-85 % (Figure 3.8) and there is no difference in β-galactosidase activity in response to copper levels (Table 3.1). Therefore, CuRE 3 alone was only able to support levels of *lacZ* activation similar to the wild-type promoter in a *mac1*Δ/*mac1*Δ strain and this is unlikely to be mediated by CaMac1p (Figure 3.3). In a triple mutant, β-galactosidase activity is the same as in the CuREs 1 & 2 mutant, showing that CuRE 3 does not contribute to activation of *lacZ* by this mutant *CaCTR1* promoter.

Mutation of CuREs 1 & 3 caused a 23 % and 60 % decrease in activity in high and low copper respectively, compared to the wild-type *CaCTR1* promoter (Figure 3.8). This decrease in activity is less severe than when just CuRE 1 is mutated, suggesting

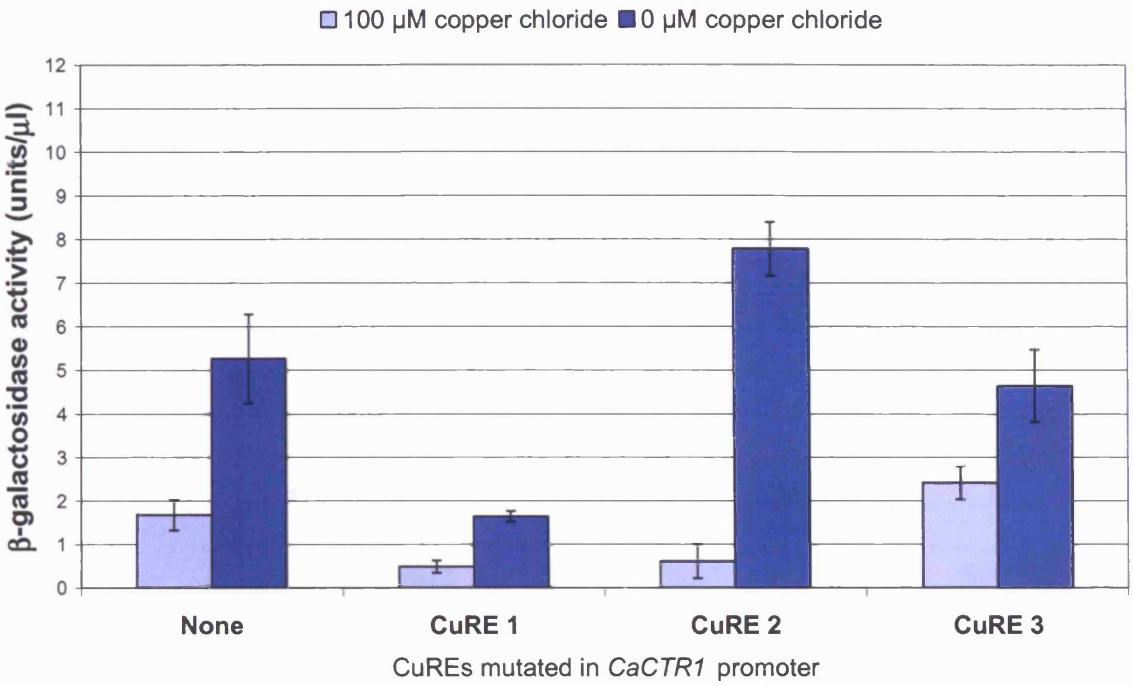


Figure 3.7 β-galactosidase activity of *CaCTR1-lacZ* fusions with mutations in one copper response element

C. albicans BWP17 (wild type) containing either wild-type *CaCTR1* promoter-*lacZ* fusion (None), or mutant *CaCTR1* promoter-*lacZ* fusions with mutations in CuRE 1, CuRE 2 or CuRE 3 were grown in high and low copper media and β-galactosidase assays were carried out as described in Materials and Methods 2.8. This figure shows the mean results of three separate experiments with error bars of one standard deviation.

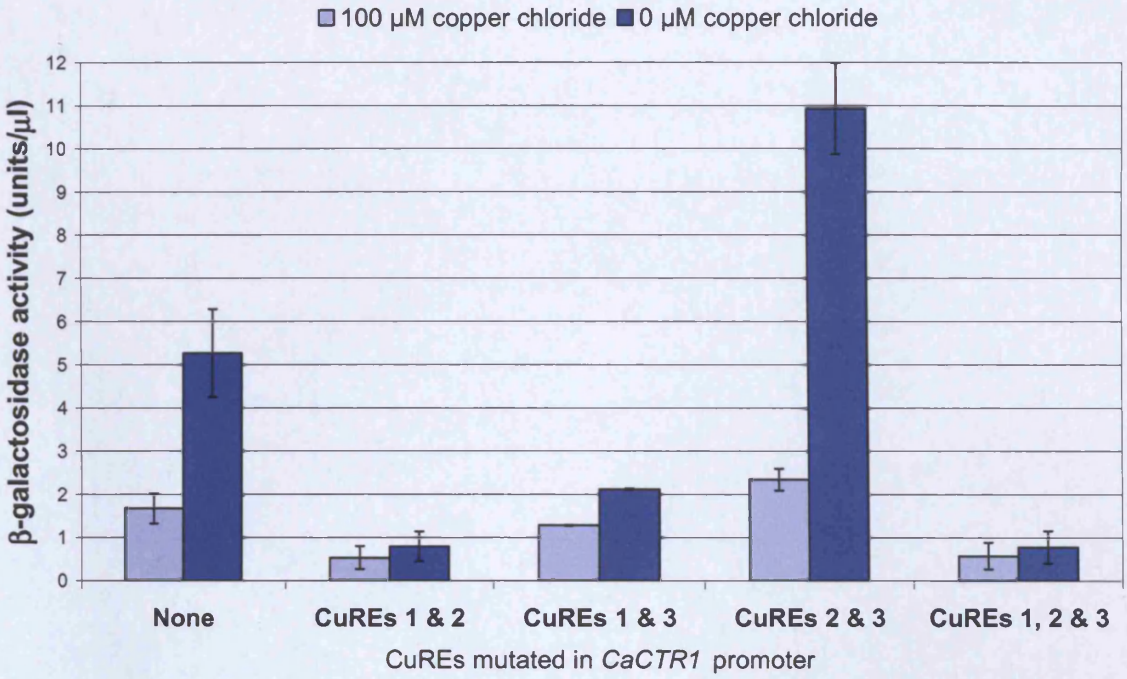


Figure 3.8 β-galactosidase activity of *CaCTR1-lacZ* fusions with mutations in two or more copper response elements

C. albicans BWP17 containing either wild-type *CaCTR1* promoter-*lacZ* fusion (None), or mutant *CaCTR1* promoter-*lacZ* fusions with mutations in the CuREs indicated were grown in high and low copper media and β-galactosidase assays were carried out as described in Materials and Methods 2.8. This figure shows the mean results of three separate experiments with error bars of one standard deviation.

that the mutation of CuRE 3 in this fusion plasmid had some effect on β-galactosidase activity of the double mutant, whereas mutation of CuRE 3 alone has no effects on β-galactosidase activity. Despite the reduced overall activity, the β-galactosidase levels of this mutant remained higher than those of the wild-type fusion plasmid in a *mac1*Δ/*mac1*Δ background strain (strain AWC1.1, Table 3.1). The CuRE 2 site alone can also mediate β-galactosidase activity in a copper-dependent manner because this mutant showed a 1.65 fold increase in activity in low copper conditions (Table 3.1).

The β-galactosidase activity of the fusion plasmid with CuREs 2 & 3 mutated was double that of a wild-type *CaCTR1* promoter in low copper conditions (Figure 3.8). In high copper conditions, activity was 40 % higher than wild type levels. In addition to an overall increase in β-galactosidase activity this mutant shows a 4.7 fold increase in activity in low copper, compared with a 3.1 fold increase in β-galactosidase activity seen with the wild-type *CaCTR1* promoter. CuRE 1 alone can therefore mediate high levels of *lacZ* expression, which are also regulated by copper concentration.

In the double mutant (CuREs 2 & 3) the mutation of CuRE 3 caused an additional increase in β-galactosidase activity of 26 % in low copper compared to the single mutant (CuRE 2). The increase in β-galactosidase activity in high copper was not statistically significant compared to the single CuRE 2 mutant, but this increased activity means that the double mutant (CuREs 2 & 3) is significantly different compared to the wild-type promoter (Table 3.1). This is similar to the effect of CuRE 3 mutation on the double mutant strain AWC6 (CuREs 1 & 3), where the double mutant shows an increase in β-galactosidase activity compared to a single CuRE 1 mutant. This suggests that mutation of CuRE 3 has an enhancing effect on β-galactosidase activity, but only when mutation of CuRE 3 is combined with mutations in other CuREs.

3.4 Construction and analysis of truncated *CaCTR1* promoter-*lacZ* fusions

Truncated *CaCTR1* promoters were constructed and ligated to plac-poly to identify important regions of the *CaCTR1* promoter and to determine if the loss of CuRE sequences from the promoter affected activation. Fragments of the *CaCTR1* promoter

containing either two, one or zero CuREs were amplified from *C. albicans* genomic DNA using forward primers that annealed 387 bp, 263 bp and 202 bp respectively upstream of the *CaCTR1* open reading frame in conjunction with the same *CaCTR1 776 R* reverse primer used to construct the full-length *CaCTR1* promoter-*lacZ* fusion. Primers *CaCTR1 387 F, CaCTR1 263 F and CaCTR1 202 F* incorporated a *Sal*I restriction site (see Table 2.7 for sequences). The PCR products and plac-poly were digested with *Sal*I and *Xma*I and the fusion plasmids pAWC9-11 were constructed by ligating the truncated *CaCTR1* promoters to the MCS of plac-poly. Plasmids pAWC9-11 were linearised by digestion with *Stu*I and transformed into *C. albicans* strain BWP17 (wild type). *CaURA3* was used as the selectable marker to identify transformants in the parental strain BWP17 (*ura3Δ/ura3Δ*). Integration of the reporter plasmids at the *CaRPS10* locus was confirmed by the presence of a 1.3 kb PCR product using primers RPS10F and URA3R as previously described.

Transformants containing the reporter plasmids pAWC1 (776 bp *CaCTR1* promoter), pAWC9 (387 bp promoter), pAWC10 (263 bp promoter) and pAWC11 (202 bp promoter) were grown in high and low copper media and β-galactosidase assays were carried out as described in Chapter 2.8. The truncated promoter of 387 bp containing only CuREs 2 and 3 showed a decrease in β-galactosidase activity of approximately 80-90 % in both high and low copper compared to the full-length wild type *CaCTR1* promoter and does not show any difference in activity in high and low copper (Figure 3.9). This result is similar to the large decrease in β-galactosidase activity seen after point mutagenesis of CuRE 1, but this point mutation did not impair the ability of the promoter to increase activation in response to low copper levels. The lack of copper responsiveness in the truncated 387 bp promoter suggests that the sequences surrounding CuRE 1 may be important for copper-regulated transcription of *CaCTR1*.

The 263 bp *CaCTR1* promoter construct containing only CuRE 3 does not show any differences in activity in high and low copper levels but shows higher β-galactosidase activity than the 387 bp promoter construct (Figure 3.9). This is consistent with the increased levels of activity observed when CuRE 2 is mutated and suggests that CuRE 2 may have some type of repressing effect on transcription. The promoter of 202 bp shows very low levels of β-galactosidase activity which is not unexpected for a very

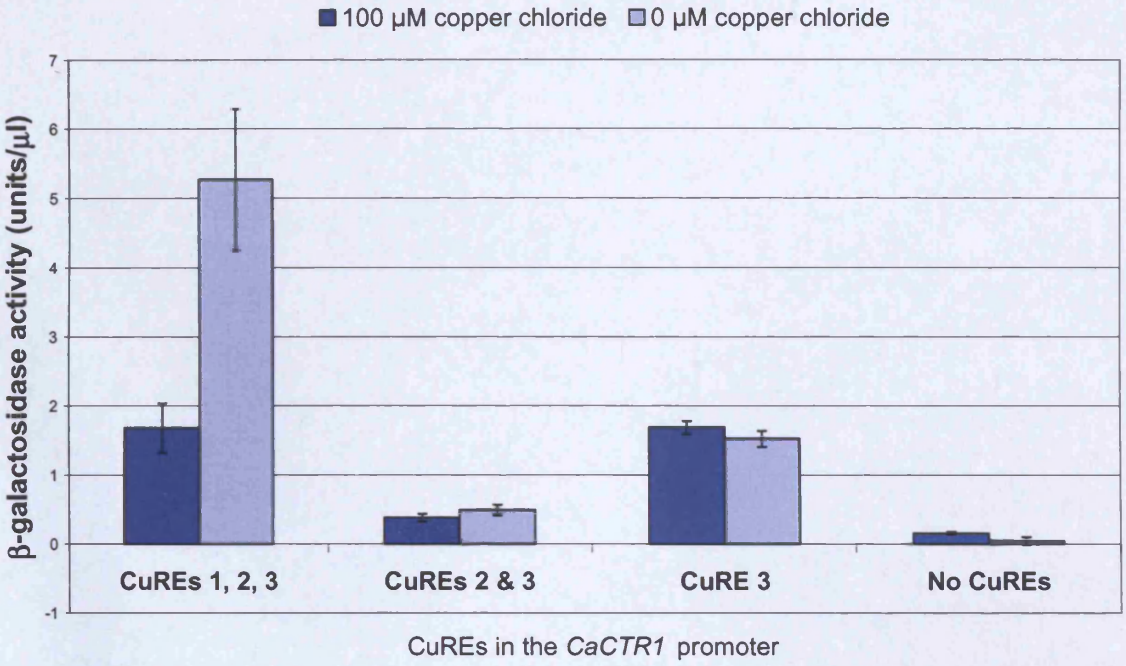


Figure 3.9 β-galactosidase activity of *CaCTR1* promoters containing different numbers of CuREs

C. albicans BWP17 containing *CaCTR1* promoter-*lacZ* fusions of different lengths were grown in high and low copper media and β-galactosidase assays were carried out as described in Materials and Methods 2.8. This figure shows the mean results of three separate experiments with error bars of one standard deviation.

short promoter without any CuREs (Figure 3.9). It is interesting to note that none of the truncated *CaCTR1* promoters show copper-dependent regulation, indicating that sequences other than the CuREs may be involved in both the overall level of transcription and in regulation in response to copper levels.

3.5 Discussion

The aim of the work described in this chapter was to determine if the putative CaMac1p binding sites identified in the *CaCTR1* promoter played a role in the regulation of *CaCTR1* by copper and CaMac1p. Using the β-galactosidase reporter constructs, it was demonstrated that *CaCTR1* promoter activity is activated three-fold under copper starvation conditions and this activation is lost in a *mac1*Δ/*mac1*Δ mutant. In fact, in the *mac1*Δ/*mac1*Δ mutant, activity is lower than in the wild type by 70 - 90 %. This is consistent with northern blot evidence that *CaMAC1* is required for copper-responsive regulation of *CaCTR1*.

The roles of the CuREs in the *CaCTR1* promoter were analysed by creating site-directed mutants of the CuRE sequences in the *CaCTR1-lacZ* fusion plasmid and measuring the β-galactosidase activity of these mutants. Mutation of CuRE 3 nearest to the ATG of *CaCTR1* does not significantly affect activation of *lacZ* by the *CaCTR1* promoter. The CuREs furthest from the ATG (CuREs 1 & 2) have more effect on β-galactosidase activity, as mutation of CuREs 1 & 2 in tandem reduces activity to less than one unit/μl and abolishes any significant difference between high and low copper conditions. This is a lower level of activity than when the *CaCTR1* promoter was introduced into the *mac1*Δ/*mac1*Δ mutant. The remaining intact CuRE 3 appears to have no effect on activation activity, and activity remains at the same level when all three CuREs are mutated. This indicates that CuRE 3 is not sufficient for activation by CaMac1p.

The reason for the low levels of activity mediated by CuRE 3 compared to the other CuREs may be because CuRE 3 is immediately preceded by TT, whereas CuREs 1 & 2 are preceded by TA. ScMac1p has been shown to bind more strongly to TATTT motifs than to TTTTT motifs in *in vitro* EMSA assays, and this is supported by mutagenesis of *ScCTR1* promoter-*lacZ* fusions (Joshi *et al.*, 1999). CuRE 3 in the

CaCTR1 promoter may therefore bind CaMac1p with lower affinity than CuREs 1 & 2, and a single binding site is therefore not sufficient for activation of *lacZ* by CaMac1p. However, the mutation of CuRE 3 does have an enhancing effect on *lacZ* activation in the double mutants shown in Figure 3.8, indicating that CaMac1p may be able to bind CuRE 3 when other binding sites are also present. Two molecules of ScMac1p form a homodimer that complexes with the promoter DNA (Joshi *et al.*, 1999), and there is a possibility that CaMac1p may participate in similar protein-protein interactions via its two predicted zinc finger domains, and this could facilitate binding to CuRE 3. Binding affinity affects the level of transactivation activity of the bound transcription factor (Joshi *et al.*, 1999), so if the CaMac1p molecules bound to CuRE 3 had a lower transactivation activity the overall β-galactosidase activity would decrease. When CuRE 3 was mutated, there was less competition for binding sites and CaMac1p molecules would bind to the more potent CuREs (1 & 2), resulting in an increase in activity.

The effect of mutating or deleting CuRE 2 is unexpected, with a subsequent increase of β-galactosidase activity compared to the wild-type promoter. A higher activation activity than the wild-type promoter may have resulted because of a number of reasons; the mutation of CuRE 2 may have formed a binding site that has a higher affinity for CaMac1p and multiple CaMac1p molecules have bound to this CuRE to activate *lacZ*; mutation of CuRE 2 may have formed a binding site for another unknown activator which is now activating *lacZ* as well as CaMac1p; or removal of CuRE 2 may have resulted in derepression of an unknown repressor molecule that has been previously repressing *CaCTR1* promoter activity. An alternative hypothesis is that CaMac1p has the most potent activation activity when bound to CuRE 1, compared to binding to any other CuREs. This theory is supported by evidence that a single mutation of CuRE 1 causes a larger decrease in β-galactosidase activity compared to mutation of CuRE 2 or CuRE 3. Activity also increases when CuRE 2 is mutated in combination with CuRE 3 and this is likely to be due to the lack of competition for binding sites by the weak binding site CuRE 3. CuRE 1 can therefore mediate high levels of β-galactosidase activity which are also copper-regulated and CuRE 1 has the most effect on activation by CaMac1p.

Mutation of CuRE 1 causes a decrease in the total promoter activity compared to a wild-type promoter, but copper-dependent regulation is maintained with a three-fold increase in low copper conditions. Mutation of CuREs 1 & 3 results in a construct with higher overall levels of β-galactosidase activity and some copper-dependent regulation, showing a 1.6 fold increase in low copper conditions. CuRE 2 is therefore sufficient for copper-responsive promoter activity and the mutation of CuRE 3 again increases total promoter activity.

Studies on ScMac1p have shown that two intact CuREs are required for copper-responsive activation of target genes including *ScCTR1* (Labbe *et al.*, 1997; Martins *et al.*, 1998). In *Schizosaccharomyces pombe*, Cuf1 has a similar role to CaMac1p and activates transcription of the copper transporter gene *ctr4*$^+$ in low copper conditions (Beaudoin & Labbe, 2001). Point mutagenesis of Cuf1 binding sites in the *ctr4*$^+$ promoter shows that a single binding site is sufficient for copper-dependent regulation of *ctr4*$^+$, although mRNA levels are lower than with a wild-type promoter (Beaudoin & Labbe, 2001). Two additional copper responsive transcription factors from *S. cerevisiae* (Ace1p) and *C. glabrata* (Amt1p) also show variation in the number of binding sites required for activation activity. One binding site is sufficient for activation of the superoxide dismutase genes, and multiple binding sites are required for activation of metallothionein genes in both organisms (Liu & Thiele, 1997). Two of the three CuREs in the *CaCTR1* promoter have clear functions in the activation of *CaCTR1* by CaMac1p and the observation that one CuRE is sufficient for copper-dependent regulation by CaMac1p suggests that CaMac1p may share characteristics with the other members of the yeast copper-responsive transcription factor family as well as to its closest homologue ScMac1p. The number of CuREs required for regulation by CaMac1p may also vary according to the target and analysis of the promoters of additional genes regulated by CaMac1p will determine whether or not this is the case.

In conclusion, the work described in this chapter demonstrates that the presence of a single copy of CuRE 1 or CuRE 2 is sufficient for copper-dependent regulation of *CaCTR1* promoter, but CuREs 1 & 2 are both required for wild-type promoter activity. CuRE 3 does not appear to be essential for copper-dependent regulation but

it is able to modulate overall levels of β-galactosidase activity. Promoter truncation experiments have also demonstrated that other sequences in the *CaCTR1* promoter are likely to be involved in copper-dependent regulation of transcription in addition to the CuREs and that the region of the promoter surrounding CuRE 2 has a repressing effect on transcription. The CuREs are therefore important for the regulation of *CaCTR1* but the promoter is complex and other regulatory elements and transcription factors are likely to be involved in the transcription of *CaCTR1* in addition to the CuREs and CaMac1p.

Chapter 4
Identification of genes with promoters containing putative CaMac1p binding sites

4.1 Introduction

The work described in Chapter 3 investigated the role of the three putative CaMac1p binding sites in the *CaCTR1* promoter using a β-galactosidase reporter strategy. Site-directed mutagenesis of the putative binding sites (CuREs) showed that the site closest to the open reading frame was not involved in copper-dependent regulation of β-galactosidase activity, however, the remaining two binding sites furthest from the open reading frame were. This evidence supports the hypothesis that *CaCTR1* is regulated by the binding of CaMac1p to two of the three CuREs in the *CaCTR1* promoter. We therefore predicted that other promoters containing two copies of the CuRE consensus sequence could also be regulated by CaMac1p. The upstream sequences of all the open reading frames in assembly 19 of the *C. albicans* genome sequence were searched for the presence of the CuRE sequence in forward and reverse orientations.

4.2 Identification of genes containing two or more putative binding sites in their promoters

The promoters of all open reading frames 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 (www.candida.bri.nrc.ca/candida). Searches for the consensus binding site sequence in both orientations were carried out, and there were 580 positive hits in the 5' – 3' direction and 540 hits in the 3' – 5' direction. The results of both searches were compared and 20 ORFs contained two or more putative binding sites in their promoters (Table 4.1). Four of these promoters, including *CaCTR1*, contained three CuREs and the remainder contained two CuREs. Seven promoters had CuREs only in the 3' – 5' direction and the remaining 13 promoters had CuREs in both orientations. CuREs were located in positions from -9 to -995 bp upstream of

Table 4.1 Results of upstream motif search for two putative CaMac1p binding sites.
The upstream motif search from the website of the *C. albicans* Research Lab at the National Research Council Canada (http://candida.bri.nrc.ca/candida) was used to search 1000 bp upstream of all the open reading frames from assembly 19 of the *C. albicans* genome sequence. The complementary consensus sequences YGMGCAAA and TTTGCKCR were used as queries and the open reading frames (Orfs) that contained two or more of these sequences are summarised in Table 4.1. Y = T/C, M = A/C, K = T/G, R = A/G. Columns 1-5 show the open reading frame number(s) in assembly 19, the gene name (if known), the position of the consensus sequences relative to the ATG and the distance between the consensus sequences. The last column shows the function of the gene and HOM denotes a function that has been assigned by the curators of the Candida Genome Database (www.candidagenome.org) based on homology to *S. cerevisiae*.

Orf no.	Gene name	YGMGCAAA	TTTGCKCR	Distance	Gene function
19.333	*CaFCY2*	-995 -292		503	Purine/cytosine permease (HOM)
19.681	*HAP43*	-928 -578		350	Transcription factor. Similar to AP-1 from *N. crassa* (HOM)
19.1971		-699 -592		107	Unknown function
19.1270	*CaFRE3*	-531	-801	270	Ferric reductase (HOM)
19.3282		-978 -465		513	Unknown function
19.3303		-808	-457	351	Carboxy-methyl transferase (HOM of *ScPPM2*)
19.3646	*CaCTR1*	-275	-397 -237	122, 38	High affinity copper permease (Marvin *et al.*, 2003)
19.4146	*CaSMD3*	-796 -187	-969	173, 609	Core snRNP protein for nucleic acid metabolism (HOM)
19.4773	*AOX2*	-776 -177 -149		599, 28	Alternative oxidase for respiratory pathway. (Huh *et al.*, 2001)
19.5599	*MDL2*	-727	-675	52	ATP-binding membrane transporter (HOM)
19.4855	*BUD31*	-794 -76		718	Bud site selection (HOM)
19.4869	*SFU1*	-823	-576	247	GATA-type transcriptional repressor (Lan *et al.*, 2004)
19.5195		-180	-191	Overlapping	Uridine-monophosphate kinase (HOM of *ScURA6*)
19.6007		-714	-601	113	Fatty acid elongation (HOM)
19.6016		-102	-966	864	Vesicle transport protein (HOM)
19.6238		-277 -122		155	Unknown function
19.7077	*CaFRE7*	-177	-132	45	Ferric reductase (HOM)
19.7078		-937	-892	45	Unknown function. Shares promoter with *CaFRE7*
19.7231	*CaFTR2*	-501 -9		492	High affinity iron permease (Ramanan & Wang, 2000)
19.7625	*PGA1*	-309	-950	641	Unknown function

the translation start site and did not show clustering in any specific region of the promoter. The distance between CuREs also showed a wide range; from two overlapping CuREs in the orf19.5195 promoter to the largest distance of 864 bp between the CuREs in the orf19.6016 promoter.

The genes identified in the search have a wide variety of roles and four of the genes in the search have functions that have been determined experimentally, including iron and copper permeases, an oxidase and a transcription factor. Eleven of the genes have been assigned functions based on their homology to *S. cerevisiae* genes and five have no known function. Five out of 20 genes are involved in copper and iron metabolism and 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 *SFU1*.

It is possible that CaMac1p regulates genes that are involved in iron and copper metabolism in a manner similar to its *S. cerevisiae* homologue ScMac1p, and three genes were selected for further analysis by northern blotting based on their putative functions in these processes (*CaFRE3, CaFRE7* and *SFU1*). 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* (Mason, 2006). *SFU1* encodes a GATA-type transcriptional repressor that represses several genes with functions in iron uptake, including the ferric reductases *FRE10*, *FRE1* and *FRE2*, iron permeases and multicopper oxidases (Lan *et al.*, 2004 and R.E. Wood, personal communication). Orf19.7078 has no known function but was also analysed because it is divergently transcribed from *CaFRE7*. These two genes also share their promoter and the two CuREs within it.

The *AOX2* and orf19.6238 genes were chosen for further analysis because they have two CuREs within their promoter 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. *CaFTR2* was not analysed further, despite its role in iron transport, because its two CuRE sequences are located at 9 bp and 501 bp from the translation start site. The CuRE at -9 bp is likely to be too close to the open reading frame to be involved in transcriptional regulation. 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). This gene is also of interest because there is no homologous gene or cyanide resistant respiration in *S. cerevisiae*, but alternative oxidases are found in higher fungi and plants (Huh & Kang, 2001).

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

Total RNA was extracted from cultures of BWP17 (wild type) and MEM-m2 (*mac1Δ/mac1Δ*) strains grown in high and low concentrations of iron and copper. Northern blots were hybridised with α-^{32}P labelled fragments of the open reading frames of all the genes selected for further analysis as described in section 4.2. All blots were also hybridised with a probe for the constitutively expressed housekeeping gene *CaACT1* as a control. The probes for northern blotting were amplified using the primers in Table 2.9. The northern blots in Figures 4.1-4.3 are representative examples of at least three repetitions for each probe used.

Expression of *AOX2* is not regulated in response to copper or iron levels in the wild type strain BWP17 or the *mac1Δ/mac1Δ* knockout strain, but transcription decreases approximately 50-fold in the *mac1Δ/mac1Δ* strain (Figure 4.1). As *AOX2* expression is not regulated by copper, it is unlikely that CaMac1p is activating this gene via the CuREs under low copper conditions in the same manner as *CaCTR1*. However, *CaMAC1* is required to maintain high levels of wild-type expression and CaMac1 protein may be affecting *AOX2* expression directly or indirectly. Direct regulation of *AOX2* by CaMac1p may occur in response to conditions other than copper and iron levels or higher copper and iron levels than the 100 μM concentrations used in this study may be required to prevent activation of *AOX2*. CaMac1p could indirectly regulate transcription of *AOX2* by activating an intermediate regulator of *AOX2*, or could interact with another activator molecule as a co-activator. CaMac1p could also

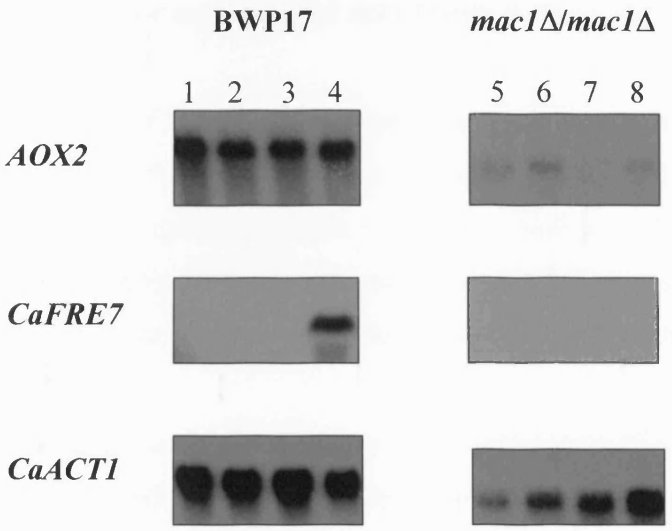


Figure 4.1 Northern blot analysis of *AOX2* and *CaFRE7* transcripts

RNA was extracted from exponentially growing cultures of BWP17 (wild type) and MEM-m2 (*mac1Δ/mac1Δ*) as described in Chapter 2.3. Denaturing formaldehyde RNA gels were northern blotted and hybridised with α^{32}P-labelled fragments of the open reading frames of *AOX2, CaFRE7* and *CaACT1*. *CaACT1* is included as a loading control. Cultures were grown in MD media as described in Chapter 2.2 with high iron (Lanes 1 & 5), low iron (Lanes 2 & 6), high copper (Lanes 3 & 7) or low copper (Lanes 4 & 8).

be involved in the regulation of the histidine kinase gene *CaSLN1*, which has been shown to be required for expression of *AOX2* (Huh & Kang, 2001).

Transcription of *CaFRE7* increases by approximately 80 times in response to low copper levels in BWP17 (Figure 4.1, Lane 4), and no transcript was detected in the absence of *CaMAC1* (Figure 4.1, Lanes 5-8). Transcription of *ScFRE7* is activated in low copper conditions by ScMac1p (Martins *et al.*, 1998), and the similar pattern of regulation in *C. albicans* indicates that *CaFRE7* is likely to be activated by CaMac1p.

Transcription of *SFU1* is decreased in low iron conditions in BWP17 (wild type) but does not appear to be affected by copper levels (Figure 4.2). Overall levels of *SFU1* transcription increase in *mac1Δ/mac1Δ*, although the transcript is still decreased approximately 3-fold in low iron conditions (Figure 4.2). In addition, *SFU1* transcription is decreased approximately 2-fold in low copper conditions in *mac1Δ/mac1Δ*. This pattern of regulation is the direct opposite of the copper-responsive CaMac1p mechanism seen so far in the regulation of *CaCTR1* and *CaFRE7*.

Transcripts for orf19.6238 and orf19.7078 did not alter in response to iron or copper levels and the same level of transcription was observed in the wild type and *mac1Δ/mac1Δ* strains (Figure 4.3). These two hypothetical open reading frames are therefore unlikely to be involved in copper and iron metabolism and are not regulated by CaMac1p. No transcript for *CaFRE3* (orf19.1270) could be detected by northern blotting in the conditions used here or during hyphal growth in YPD with 20 % (v/v) bovine calf serum. This suggests that *CaFRE3* is either not expressed in *C. albicans* or is expressed only under specific conditions that have not yet been identified.

4.4 Comparison of the Mac1p regulons in *C. albicans* and *S. cerevisiae*

An alternative method to identify genes that could potentially be regulated by CaMac1p is to identify *C. albicans* homologues of genes in the ScMac1p regulon. The ScMac1p regulon contains *CTR1, CTR3, FRE1, FRE7, YFR055w* and *YJL217w* (Gross *et al.*, 2000). The only *C. albicans* homologue of *CTR1* and *CTR3* is *CaCTR1*,

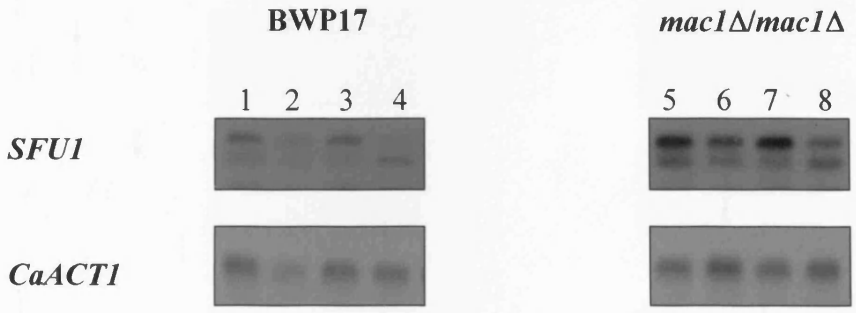


Figure 4.2 Northern blot analysis of *SFU1* transcripts

RNA was extracted from exponentially growing cultures of BWP17 (wild type) and MEM-m2 (*mac1Δ/mac1Δ*) as described in Chapter 2.3. Denaturing formaldehyde RNA gels were northern blotted and hybridised with α^{32}P-labelled fragments of the open reading frames of *SFU1* and *CaACT1*. *CaACT1* is included as a loading control. Cultures were grown in MD media as described in Chapter 2.2 with high copper (Lanes 1 & 5), low copper (Lanes 2 & 6), high iron (Lanes 3 & 7) or low iron (Lanes 4 & 8).

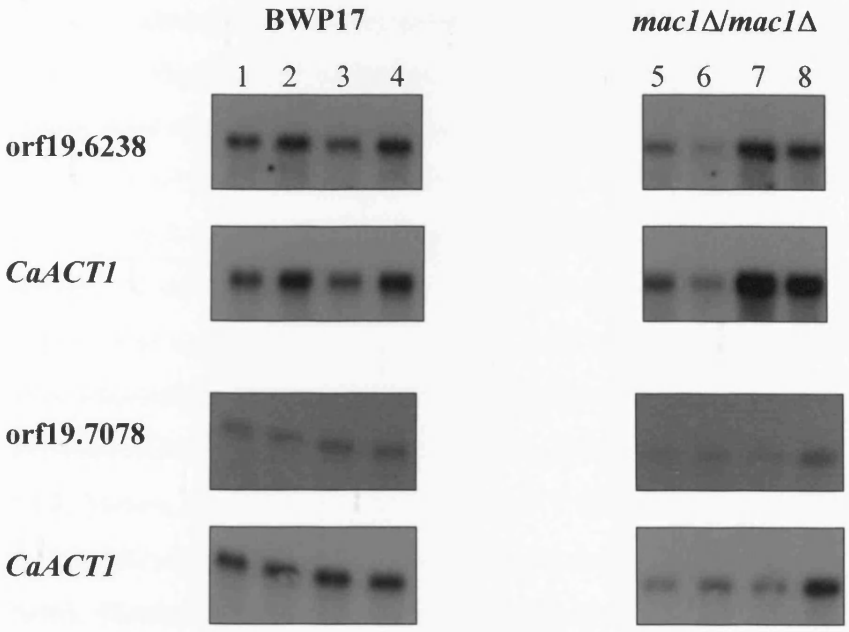


Figure 4.3 Northern blot analysis of orf19.6238 and orf19.7078 transcripts

RNA was extracted from exponentially growing cultures of BWP17 (wild type) and MEM-m2 (*mac1Δ/mac1Δ*) as described in Chapter 2.3. Denaturing formaldehyde RNA gels were northern blotted and hybridised with α^{32}P-labelled fragments of the open reading frames of orf19.6238, orf19.7078 and *CaACT1*. *CaACT1* is included as a loading control. Cultures were grown in MD media as described in Chapter 2.2 with high iron (Lanes 1 & 5), low iron (Lanes 2 & 6), high copper (Lanes 3 & 7) or low copper (Lanes 4 & 8).

which has already been shown to be regulated by CaMac1p (Marvin *et al.*, 2004). Work described in this chapter has shown that *CaFRE7* is regulated by CaMac1p in a copper-dependent manner and suggests that *CaFRE7* is likely to be the functional homologue of *ScFRE7* (Rees & Thiele, 2007). The transcript of the ferric reductase gene *CaFRE3* (orf19.1270) could not be detected by northern blot. There are a further 15 *FRE1*-like genes in the *C. albicans* genome, and a number of them have been studied in our laboratory. Previous work by our group and others has demonstrated that transcription of the major *C. albicans* ferric reductase *CaFRE10* is activated in low iron conditions, but is not affected by copper levels (Knight *et al.*, 2002; Mason, 2006). The transcription of *CaFRE2* (orf19.1264) and *CaFRE5* (orf19.1932) is not affected by copper levels or in a *mac1Δ/mac1Δ* strain (Mason, 2006). However, the transcriptional regulation of *CaFRE1* (orf19.1263) is more complex. Transcription of *CaFRE1* is increased in low iron and low copper conditions in the clinical isolate strain S/01 but transcription is not regulated by iron or copper concentrations or CaMac1p in the wild-type strain SC5314 (Hammacott *et al.*, 2000; Mason, 2006). No transcript for *CaFRE2* could be detected by northern blotting in S/01 after an exposure of the blot to X-ray film for four weeks (Hammacott, 2000), but *CaFRE2* transcript could be detected in SC5314 after an exposure of approximately one week (Mason, 2006). There may therefore be strain variation in the transcriptional regulation of *CaFRE1* and *CaFRE2*, but the vast majority of genetic and molecular research in *C. albicans* is carried out using the SC5314 strain or its derivatives. The sequence of SC5314 also reveals that no CuRE sequences are found in the promoters of *CaFRE1* or *CaFRE2* suggesting that, in this strain at least, these genes are not regulated by CaMac1p in a copper-dependent manner.

The ferric reductase genes *CaFRE10* (orf19.1415), *CaFRE6* (orf19.6138) and orf19.6139 all contain the partial CuRE sequence of TTTGCTC in their promoters at locations 427 bp, 666 bp and 743 bp upstream of the translation start site respectively, indicating the possibility of regulation by CaMac1p. A direct role in copper or iron metabolism has not yet been demonstrated for the ferric reductase-like gene orf19.6139, but microarray studies have shown that transcription of orf19.6139 is induced upon contact with macrophages and is repressed by prostaglandin (Levitin &

Whiteway, 2007; Lorenz *et al.*, 2004). A transcript could not be detected for *CaFRE6* by northern blot, so it remains unknown whether this gene is regulated by copper, iron or CaMac1p (Mason, 2006). Therefore, no *C. albicans* homologue of *ScFRE1* has yet been identified that is regulated by iron and copper levels and by CaMac1p.

The two remaining genes in the ScMac1p regulon, *YFR055w* and *YJL217w*, were identified using microarray analysis of the $MAC1^{up1}$ gain of function mutant and encode a cystathione γ-lyase and a protein of unknown function respectively (Gross *et al.*, 2000). Both of these genes were upregulated in the $MAC1^{up1}$ mutant and in the presence of the copper chelator BCS, but were downregulated in a *mac1*Δ mutant, indicating that they are activated by ScMac1p under low copper conditions (Gross *et al.*, 2000). An additional microarray of the *mac1*Δ mutant has also suggested that *YFR055w*, but not *YLJ217w*, is regulated by ScMac1p (De Freitas *et al.*, 2004). The protein sequences of *YFR055w* and *YJL217w* were used to search for homologous sequences at www.ncbi.nlm.nih.gov/BLAST. Two open reading frames in the *C. albicans* genome were identified with significant homology to *YFR055w*, but no sequences were identified with homology to *YJL217w*. *CYS3* (orf19.6402) has 26 % identity and 44 % similarity to *YFR055w,* and orf19.2092 has 25 % identity and 46 % similarity to *YFR055w*. *CYS3* encodes a putative cystathione γ-lyase and orf19.2092 encodes a putative cystathione β-lyase, and both of these enzymes are involved in the biosynthesis of cysteine. However, these genes do not contain CuREs in their promoter sequences and were not analysed further.

4.5 Discussion

The work described in this chapter suggests that the regulon of CaMac1p shares some characteristics with the regulon of ScMac1p. Transcription of *CaFRE7* is induced in low copper conditions, and this copper regulation is not seen in a *mac1*Δ/*mac1*Δ strain. This suggests that CaMac1p is activating transcription of *CaFRE7* in response to copper starvation, in a similar manner to the regulation of *ScFRE7* by ScMac1p (Martins *et al.*, 1998), giving further weight to the hypothesis that *CaFRE7* is a functional homologue of *ScFRE7* (Rees & Thiele, 2007). The transcriptional regulation of *CaFRE7* was further investigated by constructing a reporter plasmid containing a *CaFRE7* promoter-*lacZ* fusion and by mutagenesis of the putative CuRE

sequences in the *CaFRE7* promoter. The results of these experiments are described in Chapter 5.

A *C. albicans* ferric reductase gene has not yet been identified that is transcriptionally regulated by both iron and copper levels and CaMac1p in a similar manner to the *S. cerevisiae FRE1* gene (Georgatsou & Alexandraki, 1999). *ScFRE1* is regulated in response to copper by ScMac1p and in response to iron by ScAft1p, and is the only gene to be regulated by both iron and copper in this way (Georgatsou & Alexandraki, 1999). Previous work in our laboratory has identified a *C. albicans* homologue of *ScAFT1*, but *CaAFT1* does not appear to play any role in iron uptake or regulation (Mason, 2006). Therefore, an alternative transcription factor is likely to be the major regulator of iron uptake in *C. albicans*, and Sfu1p has been shown to regulate a large number of genes involved in iron uptake including ferric reductases, iron permeases and multicopper oxidases (Lan *et al.*, 2004). The evidence to date suggests that regulation of the iron and copper uptake pathways in *C. albicans* is more separate than the equivalent systems in *S. cerevisiae*. *CaFRE7* may therefore prove to be the major copper-regulated ferric/cupric reductase, whereas *CaFRE10* is the major iron-regulated ferric/cupric reductase (Knight *et al.*, 2002; Mason, 2006). However, the processes of copper and iron uptake remain linked via the absolute requirement of copper for iron uptake to take place (Knight *et al.*, 2002; Marvin *et al.*, 2004). Work is currently underway in our laboratory to construct a *CaFRE7* deletion mutant and a double deletion mutant of *CaFRE7* and *CaFRE10* (Rose Wood, personal communication). Functional analysis of these mutants will help to elucidate the relative contributions of CaFre7p and CaFre10p to the cupric and ferric reductase activity on the cell surface of *C. albicans*.

The regulation of *SFU1* and *AOX2* has suggested a number of exciting new roles for CaMac1p. Transcription of *SFU1* is not affected by copper levels in the wild type, but transcription is decreased in low iron conditions. This is in contrast to the published observation that *SFU1* transcription is not iron-responsive (Lan *et al.*, 2004). However, the low iron medium used in the study by Lan *et al.* contained 10 μM ferric ammonium sulphate, whereas the low-iron medium used in this study contained no added iron. Transcription of *SFU1* may therefore only be decreased in the lower iron concentrations used in this thesis. A decreased amount of Sfu1p in low

iron conditions would allow transcription of the genes in the iron uptake system that are repressed by Sfu1p, and facilitate the uptake of iron to alleviate iron starvation. The higher iron concentrations used by Lan *et al.* may also be the reason why results obtained in our laboratory show that *CaFRE10* transcription is repressed by Sfu1p, but the microarray study did not show any difference in *CaFRE10* expression in the *sfu1Δ/ sfu1Δ* strain (Lan *et al.*, 2004 and Rose Wood, personal communication).

Overall transcription levels of *SFU1* are increased in a *mac1Δ/mac1Δ* mutant but transcription is still decreased in low iron levels. In addition, *SFU1* transcription is decreased in low copper levels in a *mac1Δ/mac1Δ* mutant. A similar effect of *CaMAC1* deletion on transcription of *CaFRE10* is observed; in the wild type strain *CaFRE10* transcription is iron-responsive but becomes both iron- and copper-responsive in the *mac1Δ/mac1Δ* mutant (Mason, 2006). As a result of decreased *CaCTR1* expression, *mac1Δ/mac1Δ* strains are likely to be both copper and iron deficient, because *CaCTR1* transcription is required for effective iron uptake (Marvin *et al.*, 2004). This secondary iron deficiency in low copper conditions would therefore cause copper-responsive regulation of genes such as *CaFRE10* and *SFU1* in *mac1Δ/mac1Δ* strains. Upregulation of genes involved in iron uptake was also observed in *S. cerevisiae mac1Δ*, also presumably because of a secondary iron deficiency (De Freitas *et al.*, 2004). However, increased transcription of *SFU1* in the iron and copper-replete conditions in the *mac1Δ/mac1Δ* mutant is not a response to iron deficiency, as increased transcription of *SFU1* would result in a decrease in iron uptake.

An alternative explanation for the copper-responsive transcription of *SFU1* is that there is a possibility that CaMac1p is both an activator and a repressor and could be acting as a repressor to directly repress *SFU1*. The CaTup1p global repressor is also a candidate for repression of *SFU1*, and CaMac1p could bind to the *SFU1* promoter at the CuRE sequences and act as a co-repressor with CaTup1p. CaMac1p could also activate an intermediate repressor in low copper conditions and the intermediate repressor would therefore repress *SFU1* in a copper-responsive manner. Irrespective of the mechanism by which CaMac1p is affecting transcription, the interaction of copper-responsive CaMac1p with iron-responsive Sfu1p 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.

The role of CaMac1p in the transcription of *AOX2,* and its role in the respiratory chain, could not have been predicted from studies in *S. cerevisiae* because a cyanide-resistant respiratory pathway does not exist in this organism and there are no genes homologous to *AOX2* (Huh & Kang, 2001). Alternative oxidase transcription and activity is induced in low copper conditions in *Candida utilis* and the filamentous fungus *Podospora anserina* (Borghouts *et al.*, 2001; Downie & Garland, 1973). The alternative oxidase is induced because low copper conditions inactivate the cytochrome oxidase complex and the organism starts to use alternative respiratory pathways such as fermentation. However, the transcription of *AOX2* in *C. albicans* is unexpectedly decreased in the low intracellular copper conditions created in the *mac1*Δ/*mac1*Δ strain. Transcription of *AOX2* in wild type *C. albicans* is not affected by extracellular copper levels and therefore CaMac1p is unlikely to be directly activating transcription, but CaMac1p could be activating an intermediate regulator of *AOX2* or acting as a co-activator with another protein. CaMac1p may be involved in the regulation of the histidine kinase gene *CaSLN1*, which has been shown to be required for expression of *AOX2* (Huh & Kang, 2001). The alternative oxidase pathway could also be constitutively active in *C. albicans* to respond to lower copper levels experienced by this organism in the human host.

The CaMac1p regulon appears to share some similarities with the ScMac1p regulon, namely the copper transporter gene *CaCTR1* and the copper regulated ferric reductase gene *CaFRE7*. However, none of the *C. albicans* ferric reductase-like genes appear to be regulated by CaMac1p and by both iron and copper levels in a manner similar to *ScFRE1* (Georgatsou & Alexandraki, 1999). The involvement of CaMac1p in the regulation of *SFU1* and *AOX2* also shows that the Mac1p regulons in *C. albicans* and *S. cerevisiae* display key differences. The regulation of copper and iron uptake genes in these two related organisms appears to be diverged, even though many of the genes and proteins involved show a high degree of similarity.

Chapter 5
Analysis of putative CaMac1p binding sites in the promoter of *CaFRE7*

5.1 Introduction

The evidence in Chapter 4 suggests that transcription of *CaFRE7* is regulated in a similar way to the cell-surface ferric reductase gene *ScFRE7* (Martins *et al.*, 1998; Rees & Thiele, 2007). *CaFRE7* shows low homology to *S. cerevisiae* and *C. albicans* ferric reductases, with homology mostly confined to the conserved NADPH, FAD and haem binding domains, but is most similar to *ScFRE7* (Mason, 2006). The *ScFRE7* gene also appears to be less homologous to the other *S. cerevisiae* ferric reductases than they are within the gene family (Georgatsou & Alexandraki, 1999). Transcription of *CaFRE7* and *ScFRE7* is induced in low copper conditions, and transcription is regulated by CaMac1p and ScMac1p, (Chapter 4 and Martins *et al.*, 1998). The *ScFRE7* promoter contains three CuREs and at least two are required for copper-responsive expression of *ScFRE7* (Martins *et al.*, 1998). The aim of the work described in this chapter was to investigate the importance of the two CuREs in the *CaFRE7* promoter in regulation of transcription by copper levels and CaMac1p.

5.2 Construction of *CaFRE7* promoter-*lacZ* reporter strains

The region 796 bp upstream of the *CaFRE7* open reading frame was amplified from genomic DNA using the primers *CaFRE7 796 F* and *CaFRE7 796 R* (Table 2.7). The forward and reverse primers contained restriction sites for *Xho*I and *Xma*I respectively, and the PCR product and plac-poly were both digested with *Xho*I and *Xma*I. The digests were purified using MinElute PCR purification kit (Qiagen), and the *CaFRE7* promoter was ligated to plac-poly immediately upstream of the *lacZ* gene to form the reporter plasmid pAWF1.

Site-directed mutagenesis of the *CaFRE7* promoter-*lacZ* fusion plasmid

A mutagenesis strategy based on the XL-Quikchange ® Site-directed Mutagenesis Kit was used to mutate two or three bases within the CuRE sequences in the existing reporter plasmid pAWF1 and alter them from the consensus sequence of

TTTGC(T/G)C(A/G). CuREs 1 and 2 were mutated individually and in combination to give reporter plasmids AWF2-4, and Figure 5.1 shows the sequences of the wild-type and mutant *CaFRE7* promoters. The site directed mutagenesis method is described in Chapter 2.6 and used complementary primers (Table 2.10) incorporating the desired mutations to amplify both strands of pAWF1. The PCR reaction was then cleaned up using the Qiagen MinElute™ PCR purification kit and the original template plasmid was digested using *Dpn*I to target the parental *dam*-methylated plasmid DNA. Only the newly synthesised mutant plasmids were then transformed into the high-efficiency *E. coli* strain XL-10 Gold. Mutant plasmids were identified by digestion with the restriction enzymes indicated in Figure 5.1 and the mutations were confirmed by DNA sequencing.

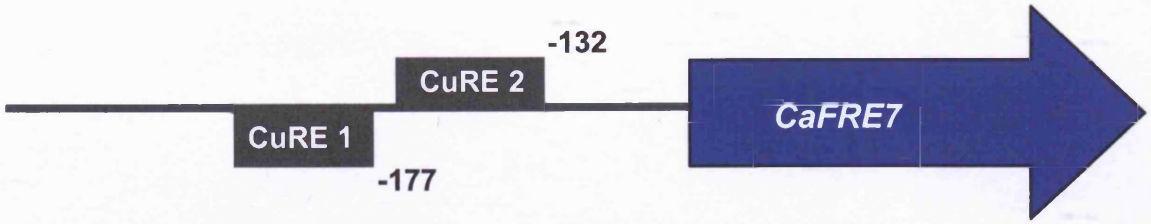
Integration of *CaFRE7* promoter-*lacZ* fusion plasmids into the *C. albicans* genome

The wild-type and mutant reporter plasmids were linearised by digestion with *Stu*I and transformed into *C. albicans* BWP17. The wild-type reporter plasmid (pAWF1) was also transformed into a *ura3*⁻ derivative of MEM-m2 (*mac1*Δ/*mac1*Δ). MEM-m2 contains a single copy of the *CaURA3* gene because *CaURA3* was used to disrupt one of the copies of *CaMAC1* in MEM-m2 (Marvin *et al.*, 2004). The single *CaURA3* allele was excised by treatment with 5-FOA, which counter-selects for uridine prototrophy. The *CaURA3* gene from plac-poly was used as the selectable marker to identify transformants in *mac1*Δ/*mac1*Δ and BWP17 (*ura3*Δ/*ura3*Δ) on SD media without uridine.

Integration of the reporter plasmids into the genome at *CaRPS10* was confirmed by PCR with primers RPS10F and URA3R, shown as red arrows in Figure 5.2, which give a product of 1.3 kb if the plasmid has integrated (data not shown). Genomic DNA from transformants was also digested with *Acc*I, resolved on a 1 % agarose gel and Southern blotted as described in Chapter 2. The blot was then hybridised with a α ^{32}P-labelled fragment of *bla* and exposed to X-ray film. The presence of one band of 6864 bp indicates that one copy of the plasmid has integrated into the *C. albicans* genome at the *CaRPS10* locus, as illustrated in Figure 5.2 a) (ii). One copy of each reporter plasmid containing the wild-type or mutant *CaFRE7* promoter was integrated

Figure 5.1 Sequences of mutant binding sites in the *CaFRE7* promoter

The figure below shows the positions of the copper response elements (CuREs) in the *CaFRE7* promoter relative to the ATG and mutated bases are shown in red. The table shows how mutant CuREs were constructed by PCR-based site-directed mutagenesis. The plasmid containing the wild-type *CaFRE7* promoter (AWF1) was amplified using primers containing the desired mutation and the additional restriction sites which are shown in the last column (see Table 2.10 for primer sequences). The methylated parental DNA was digested using *Dpn*I and the mutated plasmid DNA was transformed into *E. coli*. Mutant plasmids were screened by digestion with the additional restriction sites and the mutations were confirmed by DNA sequencing. The AWF2 plasmid containing a mutation in CuRE 1 was used as a template to construct a plasmid containing two mutated CuREs (AWF4).

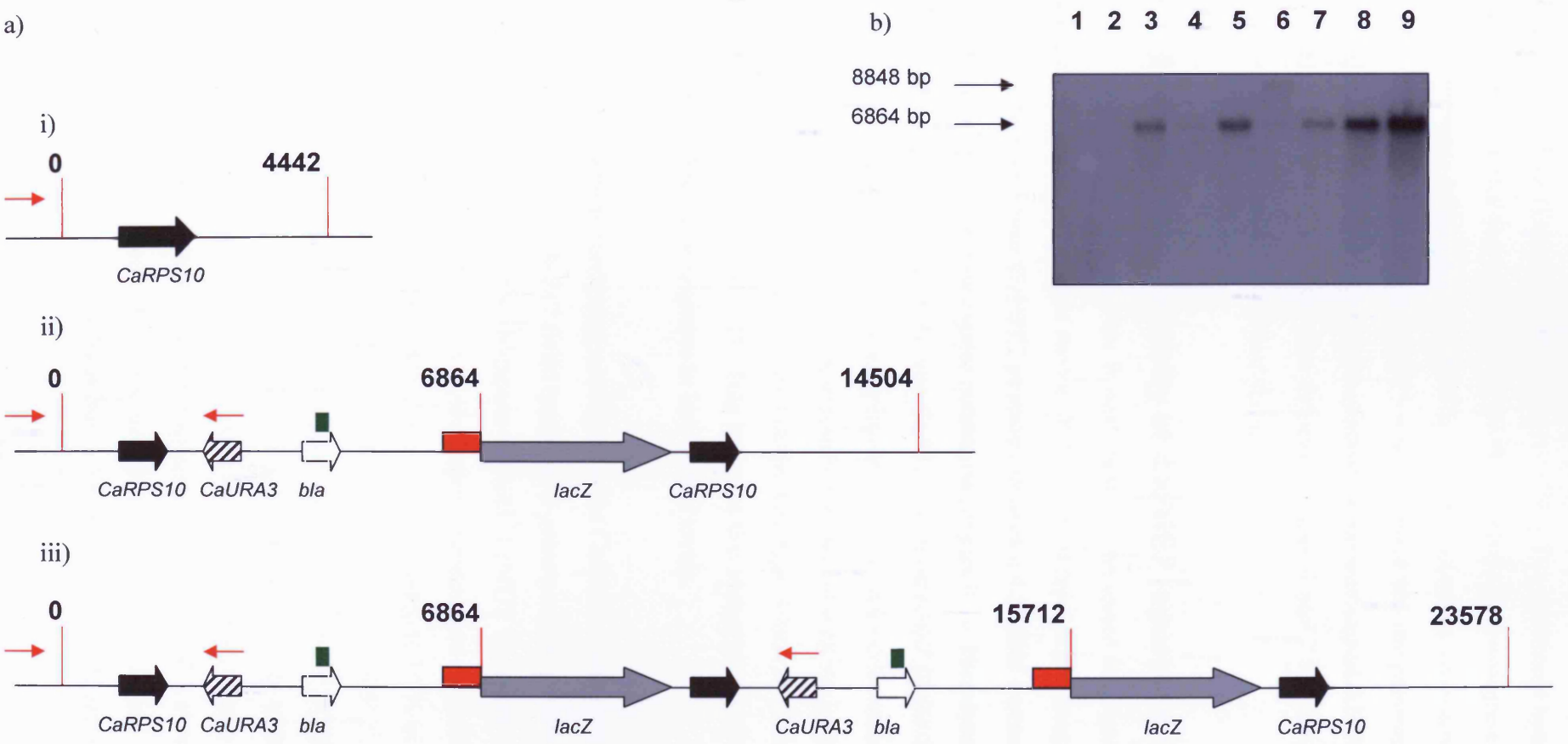


Plasmid	Template	Sequence at binding sites		Primers	Restriction sites added
		CuRE 1	CuRE 2		
pAWF1	Genomic	TGAGCAAA	TTTGCTCA	None	None
pAWF2	pAWF1	TGGCCAAA	TTTGCTCA	CaFRE7-1 F CaFRE7-1 R	*Msc*I (TGG^CCA)
pAWF3	pAWF1	TGAGCAAA	TAAGCTTA	CaFRE7-2 F CaFRE7-2 R	*Hind*III (A^AGCTT)
pAWF4	pAWF2	TGGCCAAA	TAAGCTTA	CaFRE7-2 F CaFRE7-2 R	*Msc*I, *Hind*III

Figure 5.2 Southern analysis of integration of *CaFRE7* reporter plasmids

a) The site of integration of *lacZ*-fusion plasmids at the *RPS10* locus is shown with *Acc*I restriction sites as vertical red lines. The locations where primers RPS10F and URA3R bind are shown as red arrows, the location of the *bla* probe for Southern blotting is shown in green and the *CaFRE7* promoter sequence is shown in yellow. Three possible results of transformation with *CaFRE7* promoter-*lacZ* fusion plasmids are shown (i-iii).

b) Genomic DNA was digested with *Acc*I, resolved on a 1% agarose gel and Southern blotted as described in Chapter 2. The blot was then hybridised with α ^{32}P-labelled fragment of *bla* and exposed to X-ray film. Lane 1: BWP17 (wild type) with no plasmid shows no fragments containing *bla*, as illustrated in a (i). Lane 2: *mac1*Δ/*mac1*Δ with no plasmid shows no fragments containing *bla* (i). Lane 3: BWP17 (wild type) transformed with *CaFRE7* promoter-*lacZ* fusion plasmid (pAWF1) shows one band of 6864 bp (ii) and is named AWF1. Lane 4: *mac1*Δ/*mac1*Δ transformed with *CaFRE7* promoter-*lacZ* fusion plasmid (pAWF1) shows one band of 6864 bp (ii). Lane 5: *mac1*Δ/*mac1*Δ transformed with *CaFRE7* promoter-*lacZ* fusion plasmid (pAWF1) shows one band of 6864 bp (ii) and is named AWF1.1. Lane 6: *mac1*Δ/*mac1*Δ transformed with *CaFRE7* promoter-*lacZ* fusion plasmid (pAWF1) shows one band of 6864 bp and one band of 8848 bp (iii). Lane 7: BWP17 (wild type) transformed with mutated plasmid pAWF2 shows one band of 6864 bp (ii) and is named AWF2. Lane 8: BWP17 (wild type) transformed with mutated plasmid pAWF3 shows one band of 6864 bp (ii) and is named AWF3. Lane 9: BWP17 (wild type) transformed with mutated plasmid pAWF4 shows one band of 6864 bp (ii) and is named AWF4.


a)
i)
0
4442
CaRPS10
ii)
0
6864
14504
CaRPS10
CaURA3
bla
lacZ
CaRPS10
iii)
0
6864
15712
23578
CaRPS10
CaURA3
bla
lacZ
CaURA3
bla
lacZ
CaRPS10
b)
1 2 3 4 5 6 7 8 9
8848 bp
6864 bp

into the genome of BWP17 and these transformants were named AWF1-4 and used for further analysis (Lanes 3, 7, 8, 9, Figure 5.2 b). The additional band of 8848 bp in Lane 6 indicates that two copies of the pAWF1 plasmid have integrated into the *C. albicans mac1Δ/mac1Δ* genome at the *CaRPS10* locus, as illustrated in Figure 5.2 a) (iii). However, one copy of pAWF1 was integrated into the genome of the *mac1Δ/mac1Δ* strain in Lane 5 and this transformant was named AWF1.1 and used for further analysis. No bands were detected in Lanes 1 and 2 which contained the negative controls BWP17 and *mac1Δ/mac1Δ*.

5.3 β-galactosidase activity of *CaFRE7* reporter strains

Strains containing one copy of the fusion plasmid were tested for β-galactosidase activity in high and low copper media. BWP17 (wild type) containing a reporter plasmid with a wild-type *CaFRE7* promoter showed a 4.37-fold increase in β-galactosidase activity in low copper conditions (Figure 5.3). However, β-galactosidase activity of the wild-type *CaFRE7* promoter-*lacZ* fusion in the *mac1Δ/mac1Δ* strain was significantly lower and showed no differences in high or low copper media (Figure 5.3). These results correspond with the increased *CaFRE7* transcript detected in low copper media in the wild type, which is absent in a *mac1Δ/mac1Δ* strain (Figure 4.1). This indicates that transcription of *CaFRE7* is activated by CaMac1p in response to low copper levels.

The reporter plasmids containing mutations in the *CaFRE7* promoter CuRE sequences were transformed into BWP17 (wild type) and β-galactosidase activity was measured in high and low copper media. Mutation of CuRE 1 (MUT 1) causes a 1.8-fold increase in β-galactosidase activity in low copper compared to the wild-type promoter (Figure 5.3). This is accompanied by a decrease in activity of 37 % in high copper, and resembles the alteration in β-galactosidase activity when CuRE 2 in the *CaCTR1* promoter is mutated (Figure 3.5). In contrast, mutation of CuRE 2 (MUT 2) in the *CaFRE7* promoter results in a decrease in β-galactosidase activity of 96 % in high copper, but does not significantly affect activity in low copper (Figure 5.3). Mutation of both CuREs (MUT 1 & 2) reduced β-galactosidase activity to a similar level to 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

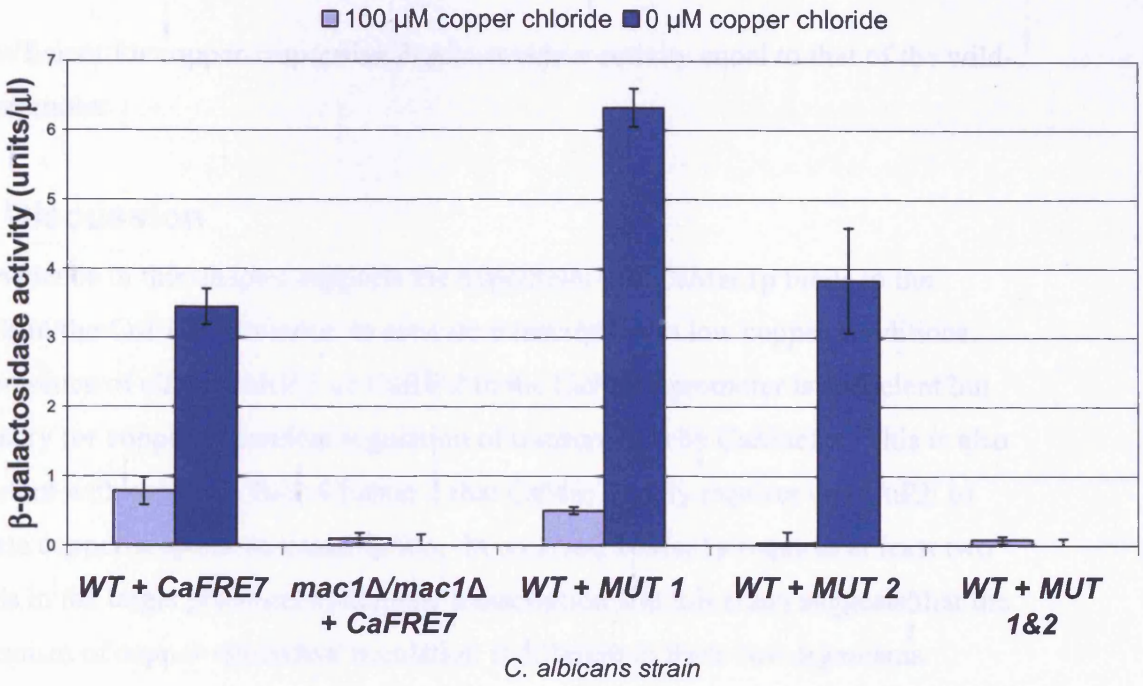


Figure 5.3 β-galactosidase activity of *CaFRE7* reporter constructs

C. albicans BWP17 (WT) and MEM-m2 (*mac1Δ/mac1Δ*) containing *CaFRE7* promoter-*lacZ* fusion plasmids were tested for β-galactosidase activity. *CaFRE7* indicates the wild-type promoter and MUT indicates which CuREs are mutated in the *CaFRE7* promoter (see Figure 5.1 for mutagenesis strategy). Reporter strains were grown in high and low copper media and β-galactosidase assays were carried out as described in Materials and Methods 2.8. This figure shows the mean of three separate experiments with error bars of one standard deviation.

and sufficient for copper-responsive β-galactosidase activity equal to that of the wild-type promoter.

5.4 Discussion

The evidence in this chapter supports the hypothesis that CaMac1p binds to the CuREs in the *CaFRE7* promoter to activate transcription in low copper conditions. The presence of either CuRE 1 or CuRE 2 in the *CaFRE7* promoter is sufficient but necessary for copper-dependent regulation of transcription by CaMac1p. This is also consistent with evidence from Chapter 3 that CaMac1p only requires one CuRE to mediate copper-responsive transcription. In contrast, ScMac1p requires at least two CuREs in the target promoter to activate transcription and this study suggests that the mechanism of copper-dependent regulation is different in these two organisms.

Transcript profiling studies have recently shown that expression of *CaFRE7* is repressed in response to prostaglandin and activated in response to engulfment by macrophages (Levitin & Whiteway, 2007; Lorenz *et al.*, 2004). Further analysis of *CaFRE7* transcription using the plac-poly reporter plasmid demonstrated that a ScMac1p binding site sequence between 120 -134 bp upstream of the open reading frame, designated CuRE 2 in the present study, is involved in the regulation of *CaFRE7* expression by prostaglandin (Levitin & Whiteway, 2007). This supports the evidence in Chapter 5 that the CuRE sequences are involved in the transcriptional regulation of *CaFRE7*. 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 by Levitin and Whiteway. Truncation of the *CaFRE7* promoter to 120 bp may also remove promoter elements in addition to the CuRE sequence that may be important for transcription. This would therefore result in a decreased amount of transcription in the absence of the CuRE sequence.

The involvement of CaMac1p in prostaglandin-mediated regulation is unexpected, and it is suggested that prostaglandin may affect CaMac1p via other transcription factors such as CaTup1p (Levitin & Whiteway, 2007). Prostaglandin-responsive transcription of *CaFRE7* was absent in a *tup1Δ/ tup1Δ* strain, but transcription of *CaFRE7* is only repressed by prostaglandin in the yeast growth phase in the wild type

(Levitin & Whiteway, 2007). The *tup1Δ/ tup1Δ* mutant is unable to grow as yeast and it is therefore likely that the growth phase-specific effect of prostaglandin on *CaFRE7* transcription is a result of the yeast growth phase rather than a direct effect of CaTup1p.

The regulation of *CaFRE7* transcription by copper and CaMac1p demonstrated in this chapter suggests that *CaFRE7* may be the homologue of *ScFRE7*. However, the transcriptional response of *CaFRE7* to host factors such as prostaglandin and macrophages suggests unique roles for CaFre7p.

Chapter 6
Transcriptional analysis of the transcriptional activator gene *CaMAC1*

6.1 Introduction

In addition to the investigation of how CaMac1p regulates other genes, we were also interested in the regulation of the *CaMAC1* gene itself. The upstream motif search described in Chapter 4 revealed that there are 1,120 CuRE sequences present in the *C. albicans* genome, including one CuRE in the promoter of the *CaMAC1* gene itself. Evidence from Chapters 3 and 5 has shown that one CuRE sequence is sufficient for copper-dependent regulation of transcription by CaMac1p. It is therefore possible that CaMac1p is able to autoregulate the *CaMAC1* gene. The related copper-responsive transcription factor Amt1p from *Candida glabrata* is autoregulated, and activates the *AMT1* gene via a single binding site when copper is at toxic levels (Zhou & Thiele, 1993). In contrast, the *S. cerevisiae MAC1* homologue is constitutively transcribed and copper-dependent regulation of ScMac1p target genes is achieved by intramolecular interaction of ScMac1p in high copper levels to inhibit the DNA binding and activation activities of the protein (Jensen & Winge, 1998; Zhu *et al.*, 1998). In very high and potentially toxic levels of copper, ScMac1p is also rapidly degraded (Zhu *et al.*, 1998). Northern blotting and reporter plasmid techniques were used to investigate the regulation of *CaMAC1* transcription.

6.2 Northern blot analysis of *CaMAC1*

Total RNA was extracted from cultures of BWP17 (wild type) and MEM-m2 (*mac1*Δ/*mac1*Δ) strains grown in high and low copper media. Northern blots were hybridised with α-^{32}P labelled fragments of the open reading frame of *CaMAC1* and the open reading frame of the constitutively expressed housekeeping gene *CaACT1* as a loading control. The probes for northern blotting were amplified using the primers in Table 2.9 and the northern blots in Figure 6.1 are representative examples of at least three repetitions. The *CaMAC1* transcript was only detected at very low levels and northern blots that contained 30 μg of total RNA required an exposure to X-ray film of at least seven days to detect any radiolabelled *CaMAC1* probe. The amount of

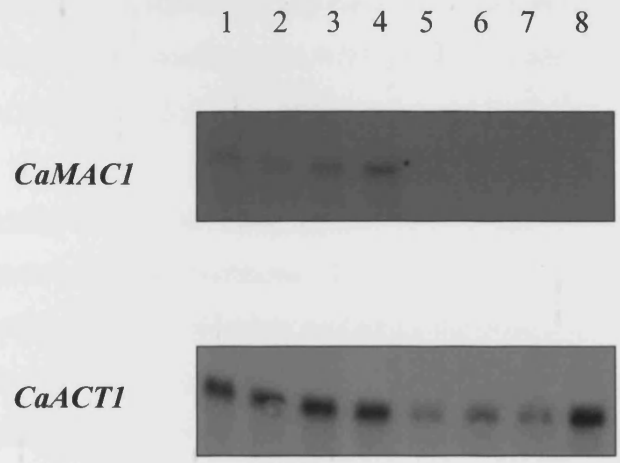


Figure 6.1 Northern blot analysis of *CaMAC1* transcripts

RNA was extracted from exponentially growing cultures of BWP17 (wild type, lanes 1-4) and MEM-m2 (*mac1*Δ/*mac1*Δ, lanes 5-8) as described in Chapter 2.3. Denaturing formaldehyde RNA gels were northern blotted and hybridised with α^{32}P-labelled fragments of the open reading frames of *CaMAC1* and *CaACT1*. *CaACT1* is included as a loading control. Cultures were grown in MD media as described in Chapter 2.2 with high iron (Lanes 1 & 5), low iron (Lanes 2 & 6), high copper (Lanes 3 & 7) or low copper (Lanes 4 & 8).

CaMAC1 transcript was approximately three times higher in low copper media compared to high copper media in the wild type. No *CaMAC1* transcript was detected in the *Camac1Δ/ Camac1Δ* mutant MEM-m2.

Transcription of *CaMAC1* therefore appears to be copper-regulated with an increase in transcript in low copper conditions. There are several possible explanations for the results obtained by northern blotting, including the possibility that *CaMAC1* transcription is activated in copper-limiting conditions by the binding of CaMac1p to the single CuRE sequence in the *CaMAC1* promoter. Transcription of *CaMAC1* may also be regulated in response to copper concentrations by another transcription factor or the *CaMAC1* mRNA may be degraded. A *CaMAC1* promoter-*lacZ* fusion plasmid was therefore constructed to test the hypothesis that *CaMAC1* transcription is autoregulated in response to copper concentrations.

6.3 Construction of *CaMAC1* promoter-*lacZ* reporter strains

The region 683 bp upstream of the *CaMAC1* open reading frame was amplified from genomic DNA using the primers *CaMAC1 683 F* and *CaMAC1 683 R* (Table 2.7). The forward and reverse primers contained restriction sites for *Xho*I and *Xma*I respectively, and the PCR product and plac-poly were both digested with *Xho*I and *Xma*I. The digests were purified using MinElute PCR purification kit (Qiagen), and the *CaMAC1* promoter was ligated to plac-poly immediately upstream of the *lacZ* gene to form the reporter plasmid pAWM1.

Site-directed mutagenesis of the *CaMAC1* promoter-*lacZ* fusion plasmid

The site-directed mutagenesis method described in Chapter 2.6 was used to mutate the existing reporter plasmid pAWM1 containing the *CaMAC1* promoter. The complementary mutagenesis primers *CaMAC1-1 F* and *CaMAC1-1 R* incorporate a change to the *CaMAC1* promoter CuRE sequence from TTTGCGCA to TTTGGCCA. These primers were used to amplify both full-length strands of pAWM1 and the PCR reaction was cleaned up using the Qiagen MinElute™ PCR purification kit before the parental plasmid DNA was digested using *Dpn*I. The mutated plasmid (pAWM2) was transformed into XL10 Gold *E. coli* and the mutations in the new plasmid were confirmed by digestion with *Hae*III and DNA sequencing.

Integration of *CaMAC1* promoter-*lacZ* fusion plasmids into the *C. albicans* genome

The fusion plasmids containing the wild-type (pAWM1) and mutant (pAWM2) *CaMAC1* promoters were digested with *Stu*I and transformed into the wild type strain BWP17 as described in Chapter 2.4. Plasmid pAWM1 was also transformed into a *ura3*⁻derivative of MEM-m2 (*mac1*Δ/*mac1*Δ) that had been treated with 5-FOA to counter-select for uridine prototrophy. Transformants were selected by uridine auxotrophy on SD media and the presence of the integrated plasmid at the *CaRPS10* locus was confirmed by PCR (data not shown). The primers RPS10F and URA3R (Table 2.8) are shown on Figure 6.2 as red arrows and will amplify a product of 1.3 kb if the reporter plasmid is present. Genomic DNA from transformants was also digested with *Acc*I, resolved on a 1 % agarose gel and Southern blotted as described in Chapter 2. The blot was then hybridised with a α ^{32}P-labelled fragment of *bla* and exposed to X-ray film. The presence of one band of 6751 bp in lanes 3, 4 and 5 indicates that one copy of the plasmid has integrated into the genome at the *CaRPS10* locus, as illustrated in Figure 6.2 a) (ii). These *C. albicans* strains were then named AWM1 (BWP17 with wild type *CaMAC1* promoter), AWM1.1 (*mac1*Δ/*mac1*Δ with wild type *CaMAC1* promoter) and AWM2 (BWP17 with mutant *CaMAC1* promoter). No bands were present in Lanes 1 and 2 containing DNA from the negative controls BWP17 and *mac1*Δ/*mac1*Δ.

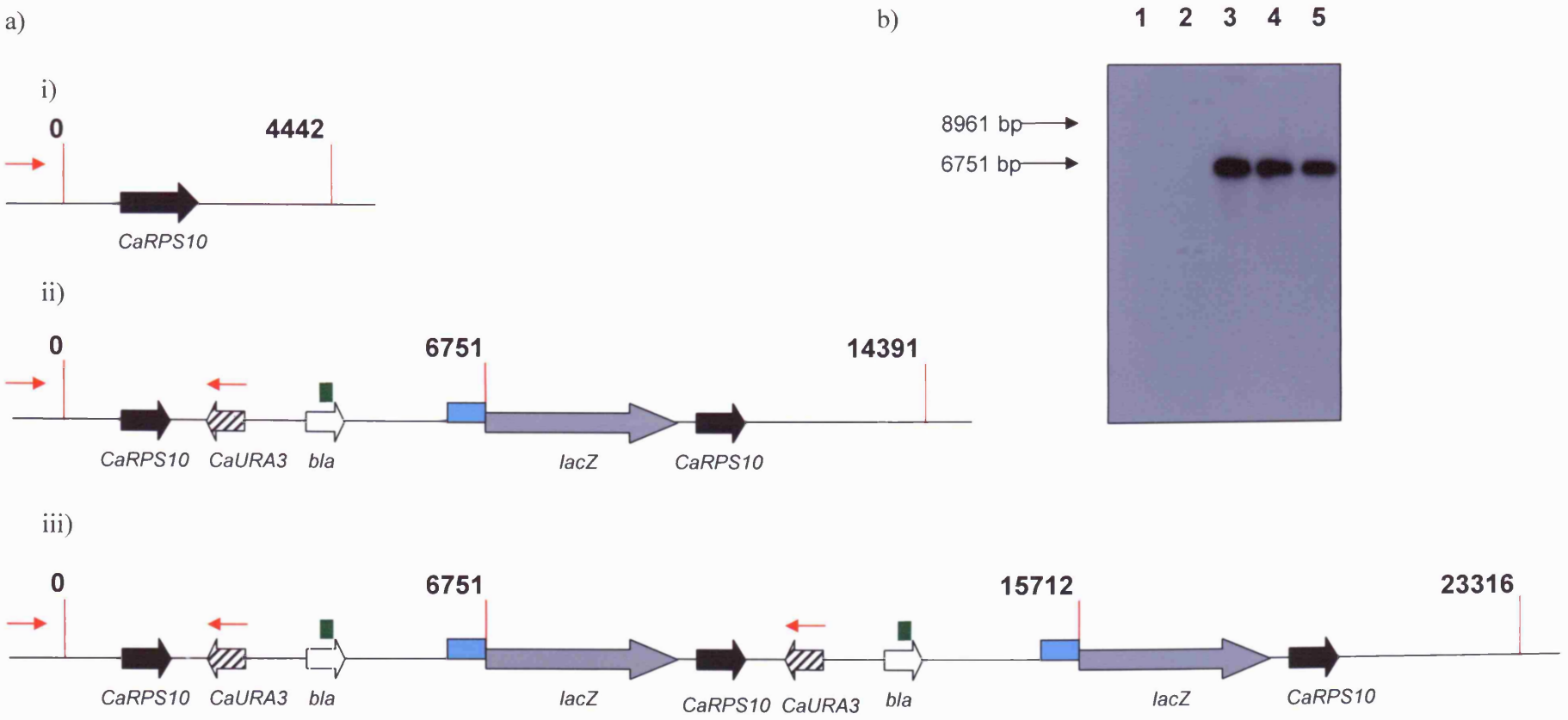
6.4 β-galactosidase activity of *CaMAC1* reporter strains

Strains AWM1, AWM1.1 and AWM2 were tested for β-galactosidase activity in high and low copper MD media as described in Chapter 2.8. BWP17 containing the wild type *CaMAC1* promoter shows a 2.8-fold increase in β-galactosidase activity in low copper conditions (Figure 6.3). This confirms northern blot data that shows *CaMAC1* transcription is induced in low copper conditions. The *mac1*Δ/*mac1*Δ strain containing the wild type *CaMAC1* promoter showed an overall decrease in β-galactosidase activity and did not show any difference in activity in high and low copper media (Figure 6.3). The mutant *CaMAC1* promoter also showed decreased β-galactosidase activity compared to the wild type promoter and there was no difference in activity in different copper levels. This evidence shows for the first time that

Figure 6.2 Southern blot analysis of *CaMAC1* promoter plasmid integration

a) The site of integration of *lacZ*-fusion plasmids at the *RPS10* locus is shown with *Acc*I restriction sites as vertical red lines. The locations where primers RPS10F and URA3R bind are shown as red arrows, the location of the *bla* probe for Southern blotting is shown in green and the *CaMAC1* promoter sequence is shown in orange. Three possible results of transformation with *CaMAC1* promoter-*lacZ* fusion plasmids are shown (i-iii).

b) Genomic DNA was digested with *Acc*I, resolved on a 1% agarose gel and Southern blotted as described in Chapter 2. The blot was then hybridised with α ^{32}P-labelled fragment of *bla* and exposed to X-ray film. Lane 1: BWP17 (wild type) with no plasmid shows no fragments containing *bla*, as illustrated in a (i). Lane 2: *mac1*Δ/*mac1*Δ with no plasmid shows no fragments containing *bla* (i). Lane 3: BWP17 (wild type) transformed with *CaMAC1* promoter-*lacZ* fusion plasmid (pAWM1) shows one band of 6751 bp (ii) and is named AWM1. Lane 4: *mac1*Δ/*mac1*Δ transformed with *CaMAC1* promoter-*lacZ* fusion plasmid (pAWM1) shows one band of 6751 bp (ii) and is named AWM1.1. Lane 5: BWP17 (wild type) transformed with mutated plasmid (pAWM2) shows one band of 6751 bp (ii) and is named AWM2. Two copies of the plasmid, as illustrated in a (iii), were not detected in any of the transformants.


a)
i)
0
4442
CaRPS10
ii)
0
6751
14391
CaRPS10
CaURA3
bla
lacZ
CaRPS10
iii)
0
6751
15712
23316
CaRPS10
CaURA3
bla
lacZ
CaRPS10
CaURA3
bla
lacZ
CaRPS10
b)
1 2 3 4 5
8961 bp
6751 bp

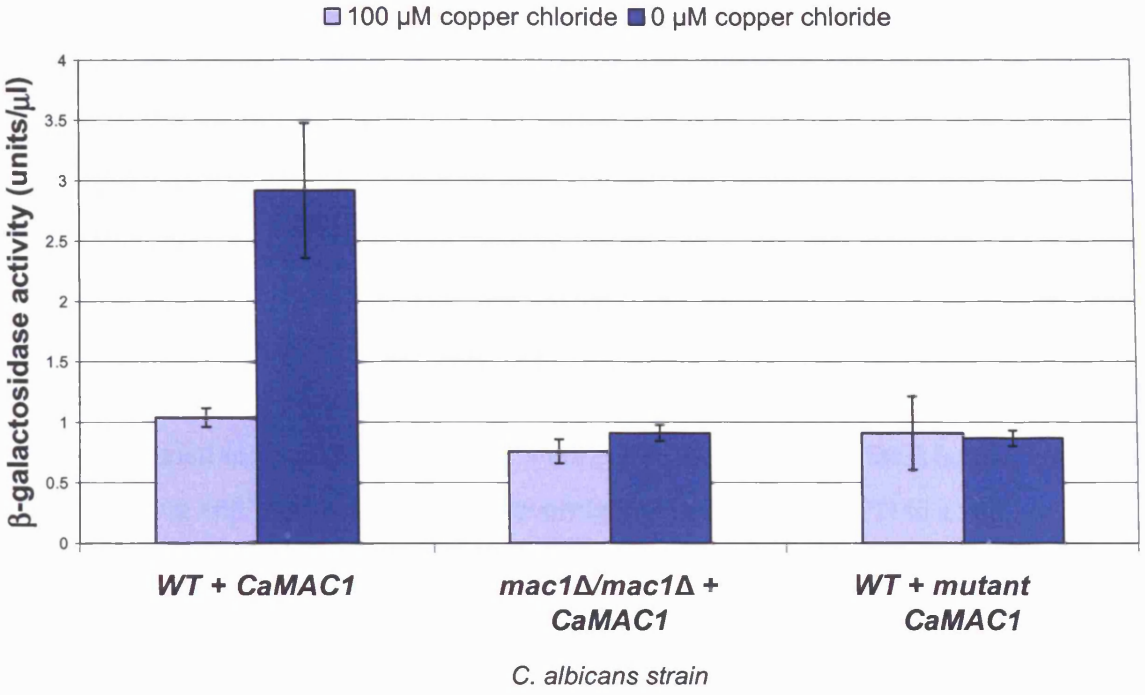


Figure 6.3 β-galactosidase activity of *CaMAC1* reporter plasmids

C. albicans BWP17 (WT) and MEM-m2 (*mac1Δ/mac1Δ*) containing *CaMAC1* promoter-*lacZ* fusion plasmids were tested for β-galactosidase activity. The mutant *CaMAC1* promoter does not contain a copper response element (CuRE). Reporter strains were grown in high and low copper media and β-galactosidase assays were carried out as described in Chapter 2.8. This figure shows the mean of three separate experiments with error bars of one standard deviation.

CaMAC1 is transcriptionally autoregulated and that the single CuRE in the *CaMAC1* promoter is involved in copper-responsive regulation by CaMac1p.

6.5 β-galactosidase activity of *lacZ* reporter strains in rich YPD medium

Reporter strains containing *lacZ* fusions with the promoters of *CaCTR1*, *CaFRE7* and *CaMAC1* were tested for β-galactosidase activity in the rich medium YPD in addition to the defined minimal medium MD. Cultures were grown in 10 ml YPD for 6 hours at 30°C with shaking and used to inoculate an overnight culture in 5 ml YPD to a cell density of approximately $3x10^4$ cells ml^{-1}. The next morning the cultures were harvested, washed and resuspended in a final volume of 1 ml sterile distilled water as previously. This suspension was used to inoculate 5ml of YPD to a cell density of $2x10^6$ cells ml^{-1}. BCS was added to YPD at a final concentration of 50 μM to chelate copper and provide a low copper medium. This culture was grown at 30°C with shaking for 5 hours until it reached exponential growth and a concentration of approximately 1 x 10^7 cells ml^{-1} and was then tested for β-galactosidase activity as described in Chapter 2.8.

Activation of *lacZ* by all three promoters tested was induced approximately three-fold after the addition of 50 μM BCS compared to growth in YPD alone (Figure 6.4). Mutation of the CuRE in the *CaMAC1* promoter abolished copper-dependent β-galactosidase activity and the level of β-galactosidase activity in the negative control containing the vector only was also extremely low (Figure 6.4). These results are consistent with northern blots and β-galactosidase assays in MD medium, demonstrating that BCS can chelate copper in rich media to the extent where the medium becomes starved of copper and copper-responsive regulation is observed. The *CaFRE7* and *CaMAC1* promoters induce the same level of β-galactosidase activity in the low copper conditions used in both MD and YPD medium, but the activity of the *CaCTR1* promoter fusion in low copper conditions is 37 % higher in low copper MD than in low copper YPD. This suggests that either additional conditions in MD may be affecting *CaCTR1* activation or that the amount of available copper in MD with 50 μM BCS is lower than in YPD with 50 μM BCS, causing additional activation of *CaCTR1* in order to relieve copper starvation.

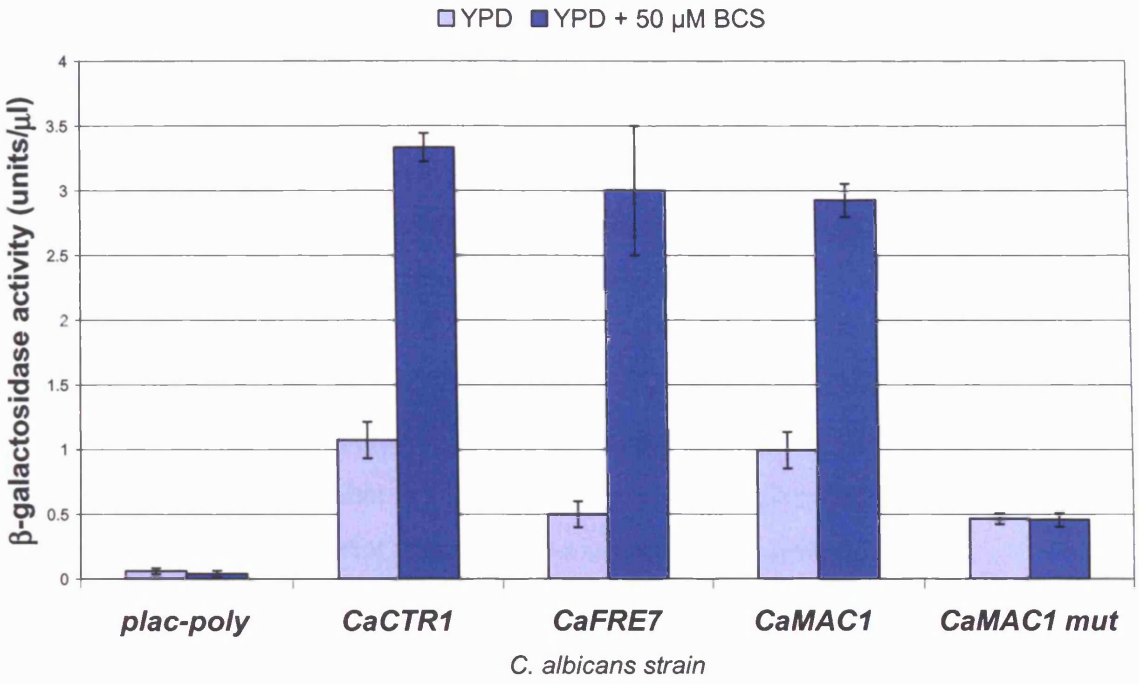


Figure 6.4 β-galactosidase activity of reporter constructs in rich YPD medium

C. albicans BWP17 (wild type) transformed with the *lacZ* reporter plasmid plac-poly containing the promoters of *CaCTR1, CaFRE7, CaMAC1* or no promoter were tested for β-galactosidase activity. The *CaMAC1 mut* promoter does not contain a copper response element (CuRE). The reporter strains were grown in YPD (high copper) or YPD containing 50 µM BCS (low copper) and β-galactosidase assays were carried out as described in Chapter 2.8. This figure shows the mean of four separate experiments with error bars of one standard deviation.

6.6 Discussion

The observation that copper-responsive transcriptional regulation is observed in a rich medium such as YPD as well as nutrient-poor minimal medium demonstrates that the response to copper is likely to be independent of other nutritional requirements and that BCS is an effective chelator of high concentrations of copper ions.

This chapter clearly shows that *CaMAC1* transcription is regulated by copper levels and transcription increases in response to copper starvation conditions. The results of the β-galactosidase assays show that the *CaMAC1* transcript is unlikely to be degraded because the *lacZ* transcript was expressed as a functional protein in a copper-dependent manner. The results of the mutagenesis experiment indicate that the copper-dependent regulation of *CaMAC1* transcription is mediated by the binding of the CaMac1 protein to the single CuRE in the promoter of *CaMAC1*. This is consistent with the results obtained in Chapters 3 and 5 that only one CuRE is required for copper-responsive activation by CaMac1p. Copper-dependent regulation of *CaMAC1* transcription is likely to have a functional role in copper homeostasis and higher levels of CaMac1p can activate transcription of *CaCTR1* and *CaFRE7* to facilitate copper uptake and alleviate copper starvation.

The copper-responsive transcription of *CaMAC1* is in contrast to the constitutive transcription of *S. cerevisiae MAC1* (Zhu *et al.*, 1998). The proteins also differ because activation by CaMac1p only requires one CuRE but ScMac1p must bind to at least two CuREs to regulate transcription of its target genes (Martins *et al.*, 1998). These key differences show that although the genes and proteins involved in copper uptake are similar in *C. albicans* and *S. cerevisiae*, their regulation appears to be diverged. Instead, the regulation of *CaMAC1* is similar to the regulation of the related copper-responsive transcription factor gene *AMT1* in *C. glabrata*. *AMT1* is autoregulated in response to copper levels by the binding of the Amt1 protein to a single binding site in the *AMT1* promoter when copper levels are high (Zhou & Thiele, 1993). The higher levels of Amt1p are then able to activate transcription of more metallothionein genes to detoxify the high levels of copper ions (Zhou & Thiele, 1993).

Although the evidence in this chapter supports the hypothesis that CaMac1p binds to the single CuRE in the *CaMAC1* promoter, the possibility remains that an alternative regulator is affecting transcription of *CaMAC1*. The base level of transcription of the *CaMAC1* promoter-*lacZ* fusion in the *mac1*Δ/*mac1*Δ strain is approximately 10-15 fold higher than the basal transcription of the *CaCTR1* and *CaFRE7* promoter fusions. There may therefore be another regulator affecting *CaMAC1* transcription in addition to CaMac1p.

Chapter 7
General Discussion

The work described in this thesis demonstrates that the transcriptional activator CaMac1p activates the transcription of the *CaCTR1* and *CaFRE7* genes in response to low copper levels. Copper response elements (CuREs) in the promoters of these genes are involved in the activation of transcription. At least one CuRE is required for transcription, but additional functional CuREs are present in the promoters of *CaCTR1* and *CaFRE7*. There is one CuRE in the *CaMAC1* promoter and this sequence is required for copper-dependent transcription of *CaMAC1*. The presence of *CaMAC1* in the genome was also required for transcription of the integrated *CaMAC1* promoter-*lacZ* reporter construct. This evidence clearly shows that a single CuRE can support copper-dependent transcriptional activation by CaMac1p and demonstrates for the first time that CaMac1p is transcriptionally autoregulated.

In contrast, optimal transcriptional activation by the *S. cerevisiae* Mac1p homologue requires the presence of two CuREs in the target promoter (Labbe *et al.*, 1997; Martins *et al.*, 1998). One of the reasons why ScMac1p requires two CuREs to be present is because it forms homodimers, and each monomer of ScMac1p binds to one CuRE sequence in the DNA (Joshi *et al.*, 1999; Serpe *et al.*, 1999). The finding that only one CuRE is required for activation by CaMac1p suggests that the formation of homodimers is not important for activation by CaMac1p, and that CaMac1p may not be able to form homodimers. Martins *et al.* (1998) did show that the presence of one CuRE in the *ScFRE7* promoter can mediate some copper-responsive transcription of a *lacZ* reporter, but the levels of activation were significantly decreased compared to wild type *ScFRE7* transcription. Transcription of *lacZ* from the *CYC1* promoter is constitutive, but the insertion of two CuREs into the *CYC1* promoter caused an increase in *lacZ* transcription in low copper conditions and a decrease in *lacZ* transcription in high copper conditions (Labbe *et al.*, 1997). However, *lacZ* transcription driven by a *CYC1* promoter containing only one CuRE was not regulated by copper levels (Labbe *et al.*, 1997). The *CTR3* promoter was also analysed by Labbe *et al.*, but the effects of a single CuRE were only tested in a truncated promoter of just 214 bp. This truncated promoter showed a doubling of activation activity after

treatment with the copper-chelator BCS, but the level of expression mediated by this promoter was again significantly reduced compared to expression from a full-length *CTR3* promoter of 1116 bp. Such extensive truncation of the *CTR3* promoter may have also removed additional sequences that are involved in transcriptional activation and regulation.

The evidence presented in this thesis supports the hypothesis that CaMac1p binds to the CuRE sequences in low copper concentrations and activates transcription of *CaCTR1*, *CaFRE7* and *CaMAC1*. Electromobility shift assays would help to determine whether CaMac1p binds directly to the CuRE sequences, or whether an intermediate transcription factor is involved. These studies would also be useful to confirm that CaMac1p transcriptionally autoregulates the *CaMAC1* gene. There is some evidence that an additional transcription factor may be involved in regulating *CaMAC1*, as basal levels of *CaMAC1-lacZ* transcription in a *mac1Δ/ mac1Δ* mutant are higher than basal levels of *CaCTR1* or *CaFRE7* transcription.

When the studies of *S. cerevisiae* CuREs are compared to the work in this thesis on the *C. albicans* CuREs, it is important to note that the promoters of *CaFRE7* and *CaMAC1* containing one CuRE are able to mediate copper-responsive transcription at the same level as a full length wild-type promoter. All of the CuREs in the *CaFRE7* and *CaMAC1* promoters are functional as single CuREs, however only CuRE 1 in the *CaCTR1* promoter is able to facilitate wild-type levels of transcription. A *CaCTR1* promoter containing only CuRE 1 has the most activity, followed by lower activity with a promoter containing CuRE 2 alone. A promoter containing only CuRE 3 has very low or no activity. The sequence surrounding the CuRE can have an effect on binding affinity and CuREs 1 and 2 are both preceded by the same TA sequence. CuRE sequences preceded by TA are bound by ScMac1p with higher affinity, and the binding of ScMac1p to promoters with this sequence results in higher activation activity (Joshi *et al.*, 1999). The CuRE 3 sequence is preceded by AT, and this may account for the lower levels of activation by CaMac1p bound to this site.

I propose the hypothesis that competition for binding sites affects the overall level of activation by CaMac1p. Higher levels of transcription are achieved when competition

for binding of CaMac1p to CuRE 1 is removed. The presence of CuRE 2 or CuRE 3 in a promoter increases competition for binding, and overall levels of transcription decrease due to the lower activation activity of CaMac1p when it is bound to these sequences. The transcription level of the *CaCTR1* gene could therefore be a combination of the activities of various CaMac1p molecules that are bound to different CuREs in the promoter.

The presence of CuREs was used to identify genes that may be regulated by CaMac1p. Although this approach had some success in identifying *CaFRE7* and *CaMAC1* as targets for CaMac1p regulation, other genes that contain CuREs in their promoters are not regulated by copper or CaMac1p. The promoter of orf19.6238 had two CuRE sequences within 300 bp of the open reading frame, making it a very likely candidate for copper-dependent regulation by CaMac1p using these criteria. However, transcript levels of orf19.6238 were not altered in response to copper or in a *Camac1Δ/ Camac1Δ* mutant. It is interesting to note that orf19.7708 is also not affected by copper or *CaMAC1*, despite being divergently transcribed from *CaFRE7* and these two genes share the same promoter and CuRE elements. This suggests that the position of the CuREs relative to the open reading frame may be a factor in determining whether they are functional, as well as the sequence of the CuRE and the surrounding DNA. This hypothesis could be tested by the construction of further *lacZ* reporters with CuREs located in different positions in the promoter and flanked by different DNA sequences.

Transcription of *AOX2* and *SFU1* is altered in the *Camac1Δ/ Camac1Δ* mutant, but is not affected in the way we would expect based on previous studies. Studies on the regulation of alternative oxidases from other fungi show that alternative oxidase transcription increases in low copper conditions (Borghouts *et al.*, 2001; Downie & Garland, 1973). This is because the activity of copper-requiring respiratory proteins such as cytochrome *c* oxidase decreases in low copper conditions and the demand for alternative electron acceptors increases. If the effects on *AOX2* transcription in the *Camac1Δ/ Camac1Δ* mutant were due to copper starvation, the level of *AOX2* transcripts would be expected to increase. Transcription of *AOX2* is not affected by copper levels in the wild type and transcription actually decreases in a *Camac1Δ/*

*Camac1*Δ mutant. This indicates that the effect of CaMac1p is not simply linked to copper levels, and CaMac1p has a specific role in the regulation of *AOX2*. However, CaMac1p is unlikely to directly regulate transcription of *AOX2* because transcription is not copper-responsive. CaMac1p could therefore influence *AOX2* transcription by activating an intermediate transcriptional activator or CaMac1p may be involved in the transcription of the histidine kinase gene *SLN1*, which is required for expression of *AOX2* (Huh & Kang, 2001).

In the wild type, the transcription of *SFU1* decreases in low iron conditions, resulting in derepression of a wide range of iron uptake genes that would help to increase iron levels (Lan *et al.*, 2004). In the *Camac1*Δ/ *Camac1*Δ mutant, *SFU1* transcription is lower in both low iron and low copper conditions. In the absence of *CaMAC1*, the extreme copper starvation conditions created by the addition of BCS results in a secondary iron starvation, due to the absolute requirement of copper for high affinity iron uptake. Transcription of SFU1 therefore appears to be regulated by both copper and iron levels in the *Camac1*Δ/ *Camac1*Δ mutant. The level of *SFU1* transcription in iron and copper-replete conditions is higher in the *Camac1*Δ/ *Camac1*Δ mutant. This does not appear to be a response to low iron or copper levels in the mutant, as copper or iron starvation would result in decreased transcription of *SFU1*. This discrepancy suggests that CaMac1p may have repressing activity as well as activating activity or CaMac1p may be indirectly repressing *SFU1* transcription via an intermediate transcription factor. It would be interesting to further investigate this link between the iron and copper regulation systems and increase our understanding of how these systems interact.

The use of CuREs was successful in identifying genes that are copper regulated by CaMac1p and it would be beneficial to employ additional strategies to continue this work to identify further genes in the CaMac1p regulon. A possible approach would be to use microarrays to compare the transcriptional profiles of wild type and *Camac1*Δ/ *Camac1*Δ cultures grown in high and low copper conditions. This would identify transcripts that are induced by low copper conditions and which are absent or significantly decreased in the *Camac1*Δ/ *Camac1*Δ mutant as members of the CaMac1p regulon. The global strategy of transcriptional profiling would also identify

genes involved in copper metabolism that are not regulated by CaMac1p, and any genes that are regulated by CaMac1p but are not involved in copper metabolism. An alternative approach would be to use chromatin immunoprecipitation, and this method would have the added advantage of identifying DNA regions where direct binding of CaMac1p takes place.

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). Three copper metabolism genes (*CaFRE7, CaCTR1* and orf19.6139) are also upregulated during engulfment 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. It would also be informative to investigate the response of *CaMAC1* and its regulon to other host factors and environments to determine when copper uptake is important in the host environment.

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. Copper-dependent regulation of *CaMAC1* transcription may help *C. albicans* to survive in both high copper and low copper environments. *C. albicans* has a high tolerance to copper, suggesting that some niches colonised by the organism may have very high copper concentrations (Riggle & Kumamoto, 2000; Weissman *et al.*, 2000). As *CaMAC1* is only expressed in 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 low copper is low, transcription of the *CaMAC1* gene is induced and this would increase the number of CaMac1p molecules available to activate target genes such as *CaCTR1*. This would help the organism to rapidly acquire copper to alleviate copper starvation.

In conclusion, the work in this thesis demonstrates that although many of the genes involved in copper uptake and metabolism are similar in *S. cerevisiae* and *C. albicans*, their mechanism of regulation appears to be diverged. The reductive iron uptake system is intrinsically linked with copper uptake, and the genes involved in iron uptake are also regulated differently from their *S. cerevisiae* homologues. Increasing evidence suggests that the ability to adapt to different niches in the human host and respond to fluctuating environmental conditions contributes to the fitness of *C. albicans* and the ability of the organism to cause disease (Brown *et al.*, 2007). Further investigation into how the copper regulatory system varies in different host niches and infection sites and during different types and stages of *Candida* infections will establish the contribution of copper uptake and regulation to the fitness and pathogenicity of *C. albicans* in the human host.

References

Aller, S. G. & Unger, V. M. (2006). Projection structure of the human copper transporter CTR1 at 6-A resolution reveals a compact trimer with a novel channel-like architecture. *Proc Natl Acad Sci U S A* **103**, 3627-3632.

Anderson, J. B. (2005). Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nat Rev Microbiol* **3**, 547-556.

Arnaud, M. B., Costanzo, M. C., Skrzypek, M. S., Binkley, G., Lane, C., Miyasato, S. R. & Sherlock, G. (2005). The Candida Genome Database (CGD), a community resource for *Candida albicans* gene and protein information. *Nucleic Acids Res* **33**, D358-363.

Arnaud, M. B., Costanzo, M. C., Skrzypek, M. S., Shah, P., Binkley, G., Lane, C., Miyasato, S. R. & Sherlock, G. (2007). Sequence resources at the Candida Genome Database. *Nucleic Acids Res* **35**, D452-456.

Askwith, C., Eide, D., Van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M. & Kaplan, J. (1994). The *FET3* gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76**, 403-410.

Askwith, C. C. & Kaplan, J. (1998). Site-directed mutagenesis of the yeast multicopper oxidase Fet3p. *J Biol Chem* **273**, 22415-22419.

Baek, Y. U., Martin, S. J. & Davis, D. A. (2006). Evidence for novel pH-dependent regulation of *Candida albicans* Rim101, a direct transcriptional repressor of the cell wall beta-glycosidase Phr2. *Eukaryot Cell* **5**, 1550-1559.

Beaudoin, J. & Labbe, S. (2001). The fission yeast copper-sensing transcription factor Cuf1 regulates the copper transporter gene expression through an Ace1/Amt1-like recognition sequence. *J Biol Chem* **276**, 15472-15480.

Beaudoin, J., Mercier, A., Langlois, R. & Labbe, S. (2003). The *Schizosaccharomyces pombe* Cuf1 is composed of functional modules from two distinct classes of copper metalloregulatory transcription factors. *J Biol Chem* **278**, 14565-14577.

Bellemare, D. R., Shaner, L., Morano, K. A., Beaudoin, J., Langlois, R. & Labbe, S. (2002). Ctr6, a vacuolar membrane copper transporter in *Schizosaccharomyces pombe*. *J Biol Chem* **277**, 46676-46686.

Bendel, C. M., Hess, D. J., Garni, R. M., Henry-Stanley, M. & Wells, C. L. (2003). Comparative virulence of *Candida albicans* yeast and filamentous forms in orally and intravenously inoculated mice. *Crit Care Med* **31**, 501-507.

Bensen, E. S., Martin, S. J., Li, M., Berman, J. & Davis, D. A. (2004). Transcriptional profiling in *Candida albicans* reveals new adaptive responses to extracellular pH and functions for Rim101p. *Mol Microbiol* **54**, 1335-1351.

Bermingham-McDonogh, O., Gralla, E. B. & Valentine, J. S. (1988). The copper, zinc-superoxide dismutase gene of *Saccharomyces cerevisiae*: cloning, sequencing, and biological activity. *Proc Natl Acad Sci U S A* **85**, 4789-4793.

Bjorklund, S. & Gustafsson, C. M. (2005). The yeast Mediator complex and its regulation. *Trends Biochem Sci* **30**, 240-244.

Blaiseau, P. L., Lesuisse, E. & Camadro, J. M. (2001). Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast. *J Biol Chem* **276**, 34221-34226.

Boeke, J. D., LaCroute, F. & Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* **197**, 345-346.

Borghouts, C., Werner, A., Elthon, T. & Osiewacz, H. D. (2001). Copper-modulated gene expression and senescence in the filamentous fungus *Podospora anserina*. *Mol Cell Biol* **21**, 390-399.

Braun, B. R., van Het Hoog, M., d'Enfert, C. & other authors (2005). A human-curated annotation of the *Candida albicans* genome. *PLoS Genet* **1**, 36-57.

Brock, J. H., Williams, P. H., Liceaga, J. & Wooldridge, K. G. (1991). Relative availability of transferrin-bound iron and cell-derived iron to aerobactin-producing and enterochelin-producing strains of *Escherichia coli* and to other microorganisms. *Infect Immun* **59**, 3185-3190.

Brown, A. J., Odds, F. C. & Gow, N. A. (2007). Infection-related gene expression in *Candida albicans*. *Curr Opin Microbiol* **10**, 307-313.

Brown, K. R., Keller, G. L., Pickering, I. J., Harris, H. H., George, G. N. & Winge, D. R. (2002). Structures of the cuprous-thiolate clusters of the Mac1 and Ace1 transcriptional activators. *Biochemistry* **41**, 6469-6476.

Care, R. S., Trevethick, J., Binley, K. M. & Sudbery, P. E. (1999). The *MET3* promoter: a new tool for *Candida albicans* molecular genetics. *Mol Microbiol* **34**, 792-798.

Carr, H. S. & Winge, D. R. (2003). Assembly of cytochrome *c* oxidase within the mitochondrion. *Acc Chem Res* **36**, 309-316.

Casareno, R. L., Waggoner, D. & Gitlin, J. D. (1998). The copper chaperone CCS directly interacts with copper/zinc superoxide dismutase. *J Biol Chem* **273**, 23625-23628.

Casas, C., Aldea, M., Espinet, C., Gallego, C., Gil, R. & Herrero, E. (1997). The *AFT1* transcriptional factor is differentially required for expression of high-affinity iron uptake genes in *Saccharomyces cerevisiae*. *Yeast* **13**, 621-637.

Cha, J. S. & Cooksey, D. A. (1991). Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. *Proc Natl Acad Sci U S A* **88**, 8915-8919.

Chibana, H., Uno, J., Cho, T. & Mikami, Y. (2005). Mutation in *IRO1* tightly linked with *URA3* gene reduces virulence of *Candida albicans*. *Microbiol Immunol* **49**, 937-939.

Cohen, A., Nelson, H. & Nelson, N. (2000). The family of *SMF* metal ion transporters in yeast cells. *J Biol Chem* **275**, 33388-33394.

Cormack, B. P., Bertram, G., Egerton, M., Gow, N. A., Falkow, S. & Brown, A. J. (1997). Yeast-enhanced green fluorescent protein (*yEGFP*)a reporter of gene expression in *Candida albicans*. *Microbiology* **143 (Pt 2)**, 303-311.

Courel, M., Lallet, S., Camadro, J. M. & Blaiseau, P. L. (2005). Direct activation of genes involved in intracellular iron use by the yeast iron-responsive transcription factor Aft2 without its paralog Aft1. *Mol Cell Biol* **25**, 6760-6771.

Crichton, R. R. & Pierre, J. L. (2001). Old iron, young copper: from Mars to Venus. *Biometals* **14**, 99-112.

d'Enfert, C., Goyard, S., Rodriguez-Arnaveilhe, S. & other authors (2005). CandidaDB: a genome database for *Candida albicans* pathogenomics. *Nucleic Acids Res* **33**, D353-357.

Dameron, C. T., Winge, D. R., George, G. N., Sansone, M., Hu, S. & Hamer, D. (1991). A copper-thiolate polynuclear cluster in the ACE1 transcription factor. *Proc Natl Acad Sci U S A* **88**, 6127-6131.

Dancis, A., Klausner, R. D., Hinnebusch, A. G. & Barriocanal, J. G. (1990). Genetic evidence that ferric reductase is required for iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**, 2294-2301.

Dancis, A., Haile, D., Yuan, D. S. & Klausner, R. D. (1994a). The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *J Biol Chem* **269**, 25660-25667.

Dancis, A., Yuan, D. S., Haile, D., Askwith, C., Eide, D., Moehle, C., Kaplan, J. & Klausner, R. D. (1994b). Molecular characterization of a copper transport protein in *S. cerevisiae*: an unexpected role for copper in iron transport. *Cell* **76**, 393-402.

De Backer, M. D., Magee, P. T. & Pla, J. (2000). Recent developments in molecular genetics of *Candida albicans*. *Annu Rev Microbiol* **54**, 463-498.

De Freitas, J. M., Kim, J. H., Poynton, H. & other authors (2004). Exploratory and confirmatory gene expression profiling of *mac1Δ*. *J Biol Chem* **279**, 4450-4458.

de Repentigny, L. (2004). Animal models in the analysis of *Candida* host-pathogen interactions. *Curr Opin Microbiol* **7**, 324-329.

de Silva, D., Davis-Kaplan, S., Fergestad, J. & Kaplan, J. (1997). Purification and characterization of Fet3 protein, a yeast homologue of ceruloplasmin. *J Biol Chem* **272**, 14208-14213.

Dhillon, N. K., Sharma, S. & Khuller, G. K. (2003). Signaling through protein kinases and transcriptional regulators in *Candida albicans*. *Crit Rev Microbiol* **29**, 259-275.

Dix, D., Bridgham, J., Broderius, M. & Eide, D. (1997). Characterization of the FET4 protein of yeast. Evidence for a direct role in the transport of iron. *J Biol Chem* **272**, 11770-11777.

Dix, D. R., Bridgham, J. T., Broderius, M. A., Byersdorfer, C. A. & Eide, D. J. (1994). The *FET4* gene encodes the low affinity Fe(II) transport protein of *Saccharomyces cerevisiae*. *J Biol Chem* **269**, 26092-26099.

Downie, J. A. & Garland, P. B. (1973). An antimycin A- and cyanide-resistant variant of *Candida utilis* arising during copper-limited growth. *Biochem J* **134**, 1051-1061.

Eck, R., Hundt, S., Hartl, A., Roemer, E. & Kunkel, W. (1999). A multicopper oxidase gene from *Candida albicans*: cloning, characterization and disruption. *Microbiology* **145 (Pt 9)**, 2415-2422.

Eide, D., Davis-Kaplan, S., Jordan, I., Sipe, D. & Kaplan, J. (1992). Regulation of iron uptake in *Saccharomyces cerevisiae*. The ferrireductase and Fe(II) transporter are regulated independently. *J Biol Chem* **267**, 20774-20781.

Eide, D. & Guarente, L. (1992). Increased dosage of a transcriptional activator gene enhances iron-limited growth of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**, 347-354.

Farrell, R. A., Thorvaldsen, J. L. & Winge, D. R. (1996). Identification of the Zn(II) site in the copper-responsive yeast transcription factor, AMT1: a conserved Zn module. *Biochemistry* **35**, 1571-1580.

Feinberg, A. P. & Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**, 6-13.

Fernandez-Arenas, E., Cabezon, V., Bermejo, C., Arroyo, J., Nombela, C., Diez-Orejas, R. & Gil, C. (2007). Integrated proteomics and genomics strategies bring new insight into *Candida albicans* response upon macrophage interaction. *Mol Cell Proteomics* **6**, 460-478.

Ferrer, J. (2000). Vaginal candidosis: epidemiological and etiological factors. *Int J Gynaecol Obstet* **71 Suppl 1**, S21-27.

Field, L. S., Luk, E. & Culotta, V. C. (2002). Copper chaperones: personal escorts for metal ions. *J Bioenerg Biomembr* **34**, 373-379.

Filler, S. G., Ibe, B. O., Ibrahim, A. S., Ghannoum, M. A., Raj, J. U. & Edwards, J. E., Jr. (1994). Mechanisms by which *Candida albicans* induces endothelial cell prostaglandin synthesis. *Infect Immun* **62**, 1064-1069.

Fragiadakis, G. S., Tzamarias, D. & Alexandraki, D. (2004). Nhp6 facilitates Aft1 binding and Ssn6 recruitment, both essential for *FRE2* transcriptional activation. *Embo J* **23**, 333-342.

Franke, K., Nguyen, M., Hartl, A., Dahse, H. M., Vogl, G., Wurzner, R., Zipfel, P. F., Kunkel, W. & Eck, R. (2006). The vesicle transport protein Vac1p is required for virulence of *Candida albicans*. *Microbiology* **152**, 3111-3121.

Garcia, M. G., O'Connor, J. E., Garcia, L. L., Martinez, S. I., Herrero, E. & del Castillo Agudo, L. (2001). Isolation of a *Candida albicans* gene, tightly linked to *URA3*, coding for a putative transcription factor that suppresses a *Saccharomyces cerevisiae aft1* mutation. *Yeast* **18**, 301-311.

Georgatsou, E. & Alexandraki, D. (1994). Two distinctly regulated genes are required for ferric reduction, the first step of iron uptake in *Saccharomyces cerevisiae*. *Mol Cell Biol* **14**, 3065-3073.

Georgatsou, E., Mavrogiannis, L. A., Fragiadakis, G. S. & Alexandraki, D. (1997). The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the copper-modulated Mac1p activator. *J Biol Chem* **272**, 13786-13792.

Georgatsou, E. & Alexandraki, D. (1999). Regulated expression of the *Saccharomyces cerevisiae* Fre1p/Fre2p Fe/Cu reductase related genes. *Yeast* **15**, 573-584.

Gillum, A. M., Tsay, E. Y. & Kirsch, D. R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. *Mol Gen Genet* **198**, 179-182.

Glerum, D. M., Shtanko, A. & Tzagoloff, A. (1996). Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. *J Biol Chem* **271**, 14504-14509.

Graden, J. A. & Winge, D. R. (1997). Copper-mediated repression of the activation domain in the yeast Mac1p transcription factor. *Proc Natl Acad Sci U S A* **94**, 5550-5555.

Gross, C., Kelleher, M., Iyer, V. R., Brown, P. O. & Winge, D. R. (2000). Identification of the copper regulon in *Saccharomyces cerevisiae* by DNA microarrays. *J Biol Chem* **275**, 32310-32316.

Gudlaugsson, O., Gillespie, S., Lee, K., Vande Berg, J., Hu, J., Messer, S., Herwaldt, L., Pfaller, M. & Diekema, D. (2003). Attributable mortality of nosocomial candidemia, revisited. *Clin Infect Dis* **37**, 1172-1177.

Haas, H. (2003). Molecular genetics of fungal siderophore biosynthesis and uptake: the role of siderophores in iron uptake and storage. *Appl Microbiol Biotechnol* **62**, 316-330.

Hammacott, J. E. (2000). Isolation and characterisation of ferric reductase genes from the pathogenic yeast *Candida albicans*. PhD thesis, *Department of Genetics*, University of Leicester, UK.

Hammacott, J. E., Williams, P. H. & Cashmore, A. M. (2000). *Candida albicans CFL1* encodes a functional ferric reductase activity that can rescue a *Saccharomyces cerevisiae fre1* mutant. *Microbiology* **146 (Pt 4)**, 869-876.

Hannula, J., Saarela, M., Dogan, B., Paatsama, J., Koukila-Kahkola, P., Pirinen, S., Alakomi, H. L., Perheentupa, J. & Asikainen, S. (2000). Comparison of virulence factors of oral *Candida dubliniensis* and *Candida albicans* isolates in healthy people and patients with chronic candidosis. *Oral Microbiol Immunol* **15**, 238-244.

Hassett, R. & Kosman, D. J. (1995). Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *J Biol Chem* **270**, 128-134.

Hassett, R., Dix, D. R., Eide, D. J. & Kosman, D. J. (2000). The Fe(II) permease Fet4p functions as a low affinity copper transporter and supports normal copper trafficking in Saccharomyces cerevisiae. *Biochem J* **351 Pt 2**, 477-484.

Hassett, R. F., Romeo, A. M. & Kosman, D. J. (1998). Regulation of high affinity iron uptake in the yeast *Saccharomyces cerevisiae*. Role of dioxygen and Fe. *J Biol Chem* **273**, 7628-7636.

Heredia, J., Crooks, M. & Zhu, Z. (2001). Phosphorylation and Cu+ coordination-dependent DNA binding of the transcription factor Mac1p in the regulation of copper transport. *J Biol Chem* **276**, 8793-8797.

Heymann, P., Ernst, J. F. & Winkelmann, G. (1999). Identification of a fungal triacetylfusarinine C siderophore transport gene (*TAF1*) in *Saccharomyces cerevisiae* as a member of the major facilitator superfamily. *Biometals* **12**, 301-306.

Heymann, P., Ernst, J. F. & Winkelmann, G. (2000a). Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **186**, 221-227.

Heymann, P., Ernst, J. F. & Winkelmann, G. (2000b). A gene of the major facilitator superfamily encodes a transporter for enterobactin (Enb1p) in *Saccharomyces cerevisiae*. *Biometals* **13**, 65-72.

Heymann, P., Gerads, M., Schaller, M., Dromer, F., Winkelmann, G. & Ernst, J. F. (2002). The siderophore iron transporter of *Candida albicans* (Sit1p/Arn1p) mediates uptake of ferrichrome-type siderophores and is required for epithelial invasion. *Infect Immun* **70**, 5246-5255.

Holzberg, M. & Artis, W. M. (1983). Hydroxamate siderophore production by opportunistic and systemic fungal pathogens. *Infect Immun* **40**, 1134-1139.

Hu, C. J., Bai, C., Zheng, X. D., Wang, Y. M. & Wang, Y. (2002). Characterization and functional analysis of the siderophore-iron transporter CaArn1p in *Candida albicans*. *J Biol Chem* **277**, 30598-30605.

Hu, S., Furst, P. & Hamer, D. (1990). The DNA and Cu binding functions of ACE1 are interdigitated within a single domain. *New Biol* **2**, 544-555.

Huang, G.-H., Nie, X-Y, Chen, J-Y (2006). CaMac1, a *Candida albicans* Copper Ion-sensing Transcription Factor, Promotes Filamentous and Invasive Growth in *Saccharomyces cerevisiae*. *Acta Biochimica et Biophysica Sinica (Shanghai)* **38**, 213-217.

Huh, W. K. & Kang, S. O. (2001). Characterization of the gene family encoding alternative oxidase from *Candida albicans*. *Biochem J* **356**, 595-604.

Hwang, C. S., Rhie, G. E., Oh, J. H., Huh, W. K., Yim, H. S. & Kang, S. O. (2002). Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. *Microbiology* **148**, 3705-3713.

Ishida, S., Lee, J., Thiele, D. J. & Herskowitz, I. (2002). Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci U S A* **99**, 14298-14302.

Ismail, A., Bedell, G. W. & Lupan, D. M. (1985). Siderophore production by the pathogenic yeast, *Candida albicans*. *Biochem Biophys Res Commun* **130**, 885-891.

Ismail, A. & Lupan, D. M. (1986). Utilization of siderophores by *Candida albicans*. *Mycopathologia* **96**, 109-113.

Jamison McDaniels, C. P., Jensen, L. T., Srinivasan, C., Winge, D. R. & Tullius, T. D. (1999). The yeast transcription factor Mac1 binds to DNA in a modular fashion. *J Biol Chem* **274**, 26962-26967.

Jensen, L. T., Posewitz, M. C., Srinivasan, C. & Winge, D. R. (1998). Mapping of the DNA binding domain of the copper-responsive transcription factor Mac1 from *Saccharomyces cerevisiae*. *J Biol Chem* **273**, 23805-23811.

Jensen, L. T. & Winge, D. R. (1998). Identification of a copper-induced intramolecular interaction in the transcription factor Mac1 from *Saccharomyces cerevisiae*. *Embo J* **17**, 5400-5408.

Jensen, L. T. & Culotta, V. C. (2002). Regulation of *Saccharomyces cerevisiae FET4* by oxygen and iron. *J Mol Biol* **318**, 251-260.

Jiang, J., Nadas, I. A., Kim, M. A. & Franz, K. J. (2005). A Mets motif peptide found in copper transport proteins selectively binds Cu(I) with methionine-only coordination. *Inorg Chem* **44**, 9787-9794.

Jones, T., Federspiel, N. A., Chibana, H. & other authors (2004). The diploid genome sequence of *Candida albicans*. *Proc Natl Acad Sci U S A* **101**, 7329-7334.

Joshi, A., Serpe, M. & Kosman, D. J. (1999). Evidence for $(Mac1p)_2$.DNA ternary complex formation in Mac1p-dependent transactivation at the *CTR1* promoter. *J Biol Chem* **274**, 218-226.

Jungmann, J., Reins, H. A., Lee, J., Romeo, A., Hassett, R., Kosman, D. & Jentsch, S. (1993). MAC1, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilization and stress resistance in yeast. *Embo J* **12**, 5051-5056.

Kalnins, A., Otto, K., Ruther, U. & Muller-Hill, B. (1983). Sequence of the *lacZ* gene of *Escherichia coli*. *Embo J* **2**, 593-597.

Kampfenkel, K., Kushnir, S., Babiychuk, E., Inze, D. & Van Montagu, M. (1995). Molecular characterization of a putative *Arabidopsis thaliana* copper transporter and its yeast homologue. *J Biol Chem* **270**, 28479-28486.

Keller, G., Gross, C., Kelleher, M. & Winge, D. R. (2000). Functional independence of the two cysteine-rich activation domains in the yeast Mac1 transcription factor. *J Biol Chem* **275**, 29193-29199.

Kellis, M., Birren, B. W. & Lander, E. S. (2004). Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature* **428**, 617-624.

Klomp, A. E., Juijn, J. A., van der Gun, L. T., van den Berg, I. E., Berger, R. & Klomp, L. W. (2003). The N-terminus of the human copper transporter 1 (hCTR1) is localized extracellularly, and interacts with itself. *Biochem J* **370**, 881-889.

Klomp, L. W., Lin, S. J., Yuan, D. S., Klausner, R. D., Culotta, V. C. & Gitlin, J. D. (1997). Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis. *J Biol Chem* **272**, 9221-9226.

Knight, S. A., Labbe, S., Kwon, L. F., Kosman, D. J. & Thiele, D. J. (1996). A widespread transposable element masks expression of a yeast copper transport gene. *Genes Dev* **10**, 1917-1929.

Knight, S. A., Lesuisse, E., Stearman, R., Klausner, R. D. & Dancis, A. (2002). Reductive iron uptake by *Candida albicans*: role of copper, iron and the *TUP1* regulator. *Microbiology* **148**, 29-40.

Knight, S. A., Vilaire, G., Lesuisse, E. & Dancis, A. (2005). Iron acquisition from transferrin by *Candida albicans* depends on the reductive pathway. *Infect Immun* **73**, 5482-5492.

Koch, K. A. & Thiele, D. J. (1996). Autoactivation by a *Candida glabrata* copper metalloregulatory transcription factor requires critical minor groove interactions. *Mol Cell Biol* **16**, 724-734.

Kuo, Y. M., Zhou, B., Cosco, D. & Gitschier, J. (2001). The copper transporter CTR1 provides an essential function in mammalian embryonic development. *Proc Natl Acad Sci U S A* **98**, 6836-6841.

Labbe, S., Zhu, Z. & Thiele, D. J. (1997). Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. *J Biol Chem* **272**, 15951-15958.

Labbe, S., Pena, M. M., Fernandes, A. R. & Thiele, D. J. (1999). A copper-sensing transcription factor regulates iron uptake genes in *Schizosaccharomyces pombe*. *J Biol Chem* **274**, 36252-36260.

Lamb, A. L., Torres, A. S., O'Halloran, T. V. & Rosenzweig, A. C. (2000). Heterodimer formation between superoxide dismutase and its copper chaperone. *Biochemistry* **39**, 14720-14727.

Lamb, A. L., Torres, A. S., O'Halloran, T. V. & Rosenzweig, A. C. (2001). Heterodimeric structure of superoxide dismutase in complex with its metallochaperone. *Nat Struct Biol* **8**, 751-755.

Lan, C. Y., Rodarte, G., Murillo, L. A., Jones, T., Davis, R. W., Dungan, J., Newport, G. & Agabian, N. (2004). Regulatory networks affected by iron availability in *Candida albicans*. *Mol Microbiol* **53**, 1451-1469.

Lane, S., Birse, C., Zhou, S., Matson, R. & Liu, H. (2001). DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. *J Biol Chem* **276**, 48988-48996.

Latchman, D. S. (1991). *Eukaryotic transcription factors*: Academic Press.

Lee, J., Prohaska, J. R., Dagenais, S. L., Glover, T. W. & Thiele, D. J. (2000). Isolation of a murine copper transporter gene, tissue specific expression and functional complementation of a yeast copper transport mutant. *Gene* **254**, 87-96.

Lee, J., Prohaska, J. R. & Thiele, D. J. (2001). Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. *Proc Natl Acad Sci U S A* **98**, 6842-6847.

Lesuisse, E., Raguzzi, F. & Crichton, R. R. (1987). Iron uptake by the yeast *Saccharomyces cerevisiae*: involvement of a reduction step. *J Gen Microbiol* **133**, 3229-3236.

Lesuisse, E. & Labbe, P. (1989). Reductive and non-reductive mechanisms of iron assimilation by the yeast *Saccharomyces cerevisiae*. *J Gen Microbiol* **135**, 257-263.

Lesuisse, E., Simon-Casteras, M. & Labbe, P. (1998). Siderophore-mediated iron uptake in *Saccharomyces cerevisiae*: the *SIT1* gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. *Microbiology* **144 (Pt 12)**, 3455-3462.

Lesuisse, E., Knight, S. A., Camadro, J. M. & Dancis, A. (2002). Siderophore uptake by *Candida albicans*: effect of serum treatment and comparison with *Saccharomyces cerevisiae*. *Yeast* **19**, 329-340.

Leuker, C. E., Hahn, A. M. & Ernst, J. F. (1992). beta-Galactosidase of *Kluyveromyces lactis* (Lac4p) as reporter of gene expression in *Candida albicans* and *C. tropicalis*. *Mol Gen Genet* **235**, 235-241.

Levitin, A. & Whiteway, M. (2007). The effect of prostaglandin E_2 on transcriptional responses of *Candida albicans*. *Microbiol Res* **162**, 201-210.

Li, D., Gurkovska, V., Sheridan, M., Calderone, R. & Chauhan, N. (2004). Studies on the regulation of the two-component histidine kinase gene *CHK1* in *Candida albicans* using the heterologous *lacZ* reporter gene. *Microbiology* **150**, 3305-3313.

Li, L., Chen, O. S., McVey Ward, D. & Kaplan, J. (2001). *CCC1* is a transporter that mediates vacuolar iron storage in yeast. *J Biol Chem* **276**, 29515-29519.

Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V. & Culotta, V. C. (1997). A role for the *Saccharomyces cerevisiae ATX1* gene in copper trafficking and iron transport. *J Biol Chem* **272**, 9215-9220.

Lin, X., Okuda, T., Holzer, A. & Howell, S. B. (2002). The copper transporter *CTR1* regulates cisplatin uptake in *Saccharomyces cerevisiae*. *Mol Pharmacol* **62**, 1154-1159.

Liu, X. D. & Thiele, D. J. (1997). Yeast metallothionein gene expression in response to metals and oxidative stress. *Methods* **11**, 289-299.

Liu, X. F., Supek, F., Nelson, N. & Culotta, V. C. (1997). Negative control of heavy metal uptake by the *Saccharomyces cerevisiae BSD2* gene. *J Biol Chem* **272**, 11763-11769.

Lo, H. J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939-949.

Lockhart, S. R., Wu, W., Radke, J. B., Zhao, R. & Soll, D. R. (2005). Increased virulence and competitive advantage of a/alpha over a/a or alpha/alpha offspring conserves the mating system of *Candida albicans*. *Genetics* **169**, 1883-1890.

Lorenz, M. C., Bender, J. A. & Fink, G. R. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3**, 1076-1087.

Magee, P. T. & Magee, B. B. (2004). Through a glass opaquely: the biological significance of mating in *Candida albicans*. *Curr Opin Microbiol* **7**, 661-665.

Mandel, M. & Higa, A. (1970). Calcium-dependent bacteriophage DNA infection. *J Mol Biol* **53**, 159-162.

Manns, J. M., Mosser, D. M. & Buckley, H. R. (1994). Production of a hemolytic factor by *Candida albicans*. *Infect Immun* **62**, 5154-5156.

Martins, L. J., Jensen, L. T., Simon, J. R., Keller, G. L. & Winge, D. R. (1998). Metalloregulation of *FRE1* and *FRE2* homologs in *Saccharomyces cerevisiae*. *J Biol Chem* **273**, 23716-23721.

Marvin, M. E., Williams, P. H. & Cashmore, A. M. (2003). The *Candida albicans CTR1* gene encodes a functional copper transporter. *Microbiology* **149**, 1461-1474.

Marvin, M. E., Mason, R. P. & Cashmore, A. M. (2004). The *CaCTR1* gene is required for high-affinity iron uptake and is transcriptionally controlled by a copper-sensing transactivator encoded by *CaMAC1*. *Microbiology* **150**, 2197-2208.

Mason, R. P. (2006). Identification and Analysis of Ferric Reductase Genes in *Candida albicans*: PhD thesis, University of Leicester, UK.

Mercier, A., Pelletier, B. & Labbe, S. (2006). A transcription factor cascade involving Fep1 and the CCAAT-binding factor Php4 regulates gene expression in response to iron deficiency in the fission yeast *Schizosaccharomyces pombe*. *Eukaryot Cell* **5**, 1866-1881.

Moore, R. E., Kim, Y. & Philpott, C. C. (2003). The mechanism of ferrichrome transport through Arn1p and its metabolism in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **100**, 5664-5669.

Morrissey, J. A., Williams, P. H. & Cashmore, A. M. (1996). *Candida albicans* has a cell-associated ferric-reductase activity which is regulated in response to levels of iron and copper. *Microbiology* **142 (Pt 3)**, 485-492.

Morschhauser, J., Michel, S. & Hacker, J. (1998). Expression of a chromosomally integrated, single-copy *GFP* gene in *Candida albicans*, and its use as a reporter of gene regulation. *Mol Gen Genet* **257**, 412-420.

Murad, A. M., Lee, P. R., Broadbent, I. D., Barelle, C. J. & Brown, A. J. (2000). Clp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16**, 325-327.

Murad, A. M., d'Enfert, C., Gaillardin, C. & other authors (2001a). Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors CaTup1, CaMig1 and CaNrg1. *Mol Microbiol* **42**, 981-993.

Murad, A. M., Leng, P., Straffon, M. & other authors (2001b). *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. *Embo J* **20**, 4742-4752.

Navarro-Garcia, F., Sanchez, M., Nombela, C. & Pla, J. (2001). Virulence genes in the pathogenic yeast *Candida albicans*. *FEMS Microbiol Rev* **25**, 245-268.

Nelissen, B., De Wachter, R. & Goffeau, A. (1997). Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **21**, 113-134.

Oberegger, H., Schoeser, M., Zadra, I., Abt, B. & Haas, H. (2001). SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in *Aspergillus nidulans*. *Mol Microbiol* **41**, 1077-1089.

Odds, F. C., Bougnoux, M. E., Shaw, D. J. & other authors (2007). Molecular phylogenetics of *Candida albicans*. *Eukaryot Cell* **6**, 1041-1052.

Odermatt, A., Suter, H., Krapf, R. & Solioz, M. (1993). Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. *J Biol Chem* **268**, 12775-12779.

Oh, K. B., Watanabe, T. & Matsuoka, H. (1999). A novel copper-binding protein with characteristics of a metallothionein from a clinical isolate of *Candida albicans*. *Microbiology* **145 (Pt 9)**, 2423-2429.

Ooi, C. E., Rabinovich, E., Dancis, A., Bonifacino, J. S. & Klausner, R. D. (1996). Copper-dependent degradation of the *Saccharomyces cerevisiae* plasma membrane copper transporter Ctr1p in the apparent absence of endocytosis. *Embo J* **15**, 3515-3523.

Pelletier, B., Beaudoin, J., Mukai, Y. & Labbe, S. (2002). Fep1, an iron sensor regulating iron transporter gene expression in *Schizosaccharomyces pombe*. *J Biol Chem* **277**, 22950-22958.

Pelletier, B., Mercier, A., Durand, M., Peter, C., Jbel, M., Beaudoin, J. & Labbe, S. (2007). Expression of *Candida albicans* Sfu1 in fission yeast complements the loss of the iron-regulatory transcription factor Fep1 and requires Tup co-repressors. *Yeast* **24**, 883-900.

Pena, M. M., Puig, S. & Thiele, D. J. (2000). Characterization of the *Saccharomyces cerevisiae* high affinity copper transporter Ctr3. *J Biol Chem* **275**, 33244-33251.

Pfaller, M. A. & Diekema, D. J. (2002). Role of sentinel surveillance of candidemia: trends in species distribution and antifungal susceptibility. *J Clin Microbiol* **40**, 3551-3557.

Pierre, J. L., Fontecave, M. & Crichton, R. R. (2002). Chemistry for an essential biological process: the reduction of ferric iron. *Biometals* **15**, 341-346.

Portnoy, M. E., Liu, X. F. & Culotta, V. C. (2000). *Saccharomyces cerevisiae* expresses three functionally distinct homologues of the nramp family of metal transporters. *Mol Cell Biol* **20**, 7893-7902.

Portnoy, M. E., Jensen, L. T. & Culotta, V. C. (2002). The distinct methods by which manganese and iron regulate the Nramp transporters in yeast. *Biochem J* **362**, 119-124.

Protchenko, O., Ferea, T., Rashford, J., Tiedeman, J., Brown, P. O., Botstein, D. & Philpott, C. C. (2001). Three cell wall mannoproteins facilitate the uptake of iron in *Saccharomyces cerevisiae*. *J Biol Chem* **276**, 49244-49250.

Pufahl, R. A., Singer, C. P., Peariso, K. L., Lin, S. J., Schmidt, P. J., Fahrni, C. J., Culotta, V. C., Penner-Hahn, J. E. & O'Halloran, T. V. (1997). Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853-856.

Puig, S., Lee, J., Lau, M. & Thiele, D. J. (2002). Biochemical and genetic analyses of yeast and human high affinity copper transporters suggest a conserved mechanism for copper uptake. *J Biol Chem* **277**, 26021-26030.

Puig, S. & Thiele, D. J. (2002). Molecular mechanisms of copper uptake and distribution. *Curr Opin Chem Biol* **6**, 171-180.

Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C. & O'Halloran, T. V. (1999). Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science* **284**, 805-808.

Raguzzi, F., Lesuisse, E. & Crichton, R. R. (1988). Iron storage in *Saccharomyces cerevisiae*. *FEBS Lett* **231**, 253-258.

Ramanan, N. & Wang, Y. (2000). A high-affinity iron permease essential for *Candida albicans* virulence. *Science* **288**, 1062-1064.

Rees, E. M., Lee, J. & Thiele, D. J. (2004). Mobilization of intracellular copper stores by the Ctr2 vacuolar copper transporter. *J Biol Chem* **279**, 54221-54229.

Rees, E. M. & Thiele, D. J. (2007). Identification of a Vacuole-associated Metalloreductase and Its Role in Ctr2-mediated Intracellular Copper Mobilization. *J Biol Chem* **282**, 21629-21638.

Richards, T. (2004). Functional analysis of *Candida albicans* copper uptake mechanism by the CaCtr1p transporter. MSc thesis, *Department of Genetics*, University of Leicester, UK.

Riggle, P. J. & Kumamoto, C. A. (2000). Role of a *Candida albicans* P1-type ATPase in resistance to copper and silver ion toxicity. *J Bacteriol* **182**, 4899-4905.

Roeder, R. G. (1996). The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem Sci* **21**, 327-335.

Rognon, B., Kozovska, Z., Coste, A. T., Pardini, G. & Sanglard, D. (2006). Identification of promoter elements responsible for the regulation of *MDR1* from *Candida albicans,* a major facilitator transporter involved in azole resistance. *Microbiology* **152**, 3701-3722.

Rupp, S. (2002). *LacZ* assays in yeast. *Methods Enzymol* **350**, 112-131.

Rustici, G., van Bakel, H., Lackner, D. H., Holstege, F. C., Wijmenga, C., Bahler, J. & Brazma, A. (2007). Global transcriptional responses of fission and budding yeast to changes in copper and iron levels: a comparative study. *Genome Biol* **8**, R73.

Rutherford, J. C., Jaron, S., Ray, E., Brown, P. O. & Winge, D. R. (2001). A second iron-regulatory system in yeast independent of Aft1p. *Proc Natl Acad Sci U S A* **98**, 14322-14327.

Rutherford, J. C., Jaron, S. & Winge, D. R. (2003). Aft1p and Aft2p mediate iron-responsive gene expression in yeast through related promoter elements. *J Biol Chem* **278**, 27636-27643.

Santos, M. A. & Tuite, M. F. (1995). The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans. Nucleic Acids Res* **23**, 1481-1486.

Schaible, U. E. & Kaufmann, S. H. (2004). Iron and microbial infection. *Nat Rev Microbiol* **2**, 946-953.

Schmitt, M. E., Brown, T. A. & Trumpower, B. L. (1990). A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae. Nucleic Acids Res* **18**, 3091-3092.

Schrettl, M., Winkelmann, G. & Haas, H. (2004). Ferrichrome in *Schizosaccharomyces pombe*--an iron transport and iron storage compound. *Biometals* **17**, 647-654.

Schroeder, C. J., Robert, C., Lenzen, G., McKay, L. L. & Mercenier, A. (1991). Analysis of the *lacZ* sequences from two *Streptococcus thermophilus* strains: comparison with the *Escherichia coli* and *Lactobacillus bulgaricus* beta-galactosidase sequences. *J Gen Microbiol* **137**, 369-380.

Serpe, M., Joshi, A. & Kosman, D. J. (1999). Structure-function analysis of the protein-binding domains of Mac1p, a copper-dependent transcriptional activator of copper uptake in *Saccharomyces cerevisiae. J Biol Chem* **274**, 29211-29219.

Sickmann, A., Reinders, J., Wagner, Y. & other authors (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci U S A* **100**, 13207-13212.

Sinani, D., Adle, D. J., Kim, H. & Lee, J. (2007). Distinct Mechanisms for Ctr1-mediated Copper and Cisplatin Transport. *J Biol Chem* **282**, 26775-26785.

Singh, A., Kaur, N. & Kosman, D. J. (2007). The metalloreductase Fre6p in Fe-efflux from the yeast vacuole. *J Biol Chem.*

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503-517.

Spizzo, T., Byersdorfer, C., Duesterhoeft, S. & Eide, D. (1997). The yeast *FET5* gene encodes a *FET3*-related multicopper oxidase implicated in iron transport. *Mol Gen Genet* **256**, 547-556.

Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D. & Dancis, A. (1996). A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science* **271**, 1552-1557.

Sudbery, P., Gow, N. & Berman, J. (2004). The distinct morphogenic states of *Candida albicans. Trends Microbiol* **12**, 317-324.

Sweet, S. P. & Douglas, L. J. (1991). Effect of iron concentration on siderophore synthesis and pigment production by *Candida albicans. FEMS Microbiol Lett* **64**, 87-91.

Swoboda, R. K., Broadbent, I. D., Bertram, G., Budge, S., Gooday, G. W., Gow, N. A. & Brown, A. J. (1995). Structure and regulation of a *Candida albicans RP10* gene which encodes an immunogenic protein homologous to *Saccharomyces cerevisiae* ribosomal protein 10. *J Bacteriol* **177**, 1239-1246.

Szczypka, M. S., Zhu, Z., Silar, P. & Thiele, D. J. (1997). *Saccharomyces cerevisiae* mutants altered in vacuole function are defective in copper detoxification and iron-responsive gene transcription. *Yeast* **13**, 1423-1435.

Thomas, D. A. (2003). Analysis of the genes involved in copper transport in *Candida albicans*. MSc thesis, *Department of Genetics*, University of Leicester, UK.

Thorvaldsen, J. L., Sewell, A. K., McCowen, C. L. & Winge, D. R. (1993). Regulation of metallothionein genes by the ACE1 and AMT1 transcription factors. *J Biol Chem* **268**, 12512-12518.

Thorvaldsen, J. L., Sewell, A. K., Tanner, A. M., Peltier, J. M., Pickering, I. J., George, G. N. & Winge, D. R. (1994). Mixed Cu^{+} and Zn^{2+} coordination in the DNA-binding domain of the AMT1 transcription factor from *Candida glabrata. Biochemistry* **33**, 9566-9577.

Tirosh, I., Berman, J. & Barkai, N. (2007). The pattern and evolution of yeast promoter bendability. *Trends Genet* **23**, 318-321.

Tortorano, A. M., Peman, J., Bernhardt, H. & other authors (2004). Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. *Eur J Clin Microbiol Infect Dis* **23**, 317-322.

Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itoh, K., Nakashima, R., Yaono, R. & Yoshikawa, S. (1995). Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 A. *Science* **269**, 1069-1074.

Turski, M. L. & Thiele, D. J. (2007). Drosophila Ctr1A functions as a copper transporter essential for development. *J Biol Chem* **282**, 24017-24026.

Ueta, R., Fujiwara, N., Iwai, K. & Yamaguchi-Iwai, Y. (2007). Mechanism underlying the iron-dependent nuclear export of the iron-responsive transcription factor Aft1p in *Saccharomyces cerevisiae. Mol Biol Cell* **18**, 2980-2990.

Uhl, M. A. & Johnson, A. D. (2001). Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans. Microbiology* **147**, 1189-1195.

Urbanowski, J. L. & Piper, R. C. (1999). The iron transporter Fth1p forms a complex with the Fet5 iron oxidase and resides on the vacuolar membrane. *J Biol Chem* **274**, 38061-38070.

Urbanski, N. K. & Beresewicz, A. (2000). Generation of *OH initiated by interaction of Fe^{2+} and Cu^{+} with dioxygen; comparison with the Fenton chemistry. *Acta Biochim Pol* **47**, 951-962.

van Bakel, H., Strengman, E., Wijmenga, C. & Holstege, F. C. (2005). Gene expression profiling and phenotype analyses of *S. cerevisiae* in response to changing copper reveals six genes with new roles in copper and iron metabolism. *Physiol Genomics* **22**, 356-367.

van het Hoog, M., Rast, T. J., Martchenko, M. & other authors (2007). Assembly of the *Candida albicans* genome into sixteen supercontigs aligned on the eight chromosomes. *Genome Biol* **8**, R52.

Van Ho, A., Ward, D. M. & Kaplan, J. (2002). Transition metal transport in yeast. *Annu Rev Microbiol* **56**, 237-261.

Voisard, C., Wang, J., McEvoy, J. L., Xu, P. & Leong, S. A. (1993). *urbs1*, a gene regulating siderophore biosynthesis in *Ustilago maydis*, encodes a protein similar to the erythroid transcription factor GATA-1. *Mol Cell Biol* **13**, 7091-7100.

Wahl, G. M., Stern, M. & Stark, G. R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc Natl Acad Sci U S A* **76**, 3683-3687.

Weissman, Z., Berdicevsky, I., Cavari, B. Z. & Kornitzer, D. (2000). The high copper tolerance of *Candida albicans* is mediated by a P-type ATPase. *Proc Natl Acad Sci U S A* **97**, 3520-3525.

Weissman, Z., Shemer, R. & Kornitzer, D. (2002). Deletion of the copper transporter *CaCCC2* reveals two distinct pathways for iron acquisition in *Candida albicans*. *Mol Microbiol* **44**, 1551-1560.

White, T. C., Andrews, L. E., Maltby, D. & Agabian, N. (1995). The "universal" leucine codon CTG in the secreted aspartyl proteinase 1 (SAP1) gene of *Candida albicans* encodes a serine in vivo. *J Bacteriol* **177**, 2953-2955.

Wickerham, L. J. (1946). A Critical Evaluation of the Nitrogen Assimilation Tests Commonly Used in the Classification of Yeasts. *J Bacteriol* **52**, 293-301.

Wilson, R. B., Davis, D. & Mitchell, A. P. (1999). Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* **181**, 1868-1874.

Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P. & Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* **39**, 309-317.

Xiao, Z. & Wedd, A. G. (2002). A C-terminal domain of the membrane copper pump Ctr1 exchanges copper(I) with the copper chaperone Atx1. *Chem Commun (Camb)*, 588-589.

Xiao, Z., Loughlin, F., George, G. N., Howlett, G. J. & Wedd, A. G. (2004). C-terminal domain of the membrane copper transporter Ctr1 from *Saccharomyces cerevisiae* binds four Cu(I) ions as a cuprous-thiolate polynuclear cluster: sub-femtomolar Cu(I) affinity of three proteins involved in copper trafficking. *J Am Chem Soc* **126**, 3081-3090.

Xu, Z., Zhang, L. X., Zhang, J. D., Cao, Y. B., Yu, Y. Y., Wang, D. J., Ying, K., Chen, W. S. & Jiang, Y. Y. (2006). cDNA microarray analysis of differential gene expression and regulation in clinically drug-resistant isolates of *Candida albicans* from bone marrow transplanted patients. *Int J Med Microbiol* **296**, 421-434.

Yamaguchi-Iwai, Y., Dancis, A. & Klausner, R. D. (1995). AFT1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *Embo J* **14**, 1231-1239.

Yamaguchi-Iwai, Y., Stearman, R., Dancis, A. & Klausner, R. D. (1996). Iron-regulated DNA binding by the AFT1 protein controls the iron regulon in yeast. *Embo J* **15**, 3377-3384.

Yamaguchi-Iwai, Y., Serpe, M., Haile, D., Yang, W., Kosman, D. J., Klausner, R. D. & Dancis, A. (1997). Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of *FRE1* and *CTR1*. *J Biol Chem* **272**, 17711-17718.

Yamaguchi-Iwai, Y., Ueta, R., Fukunaka, A. & Sasaki, R. (2002). Subcellular localization of Aft1 transcription factor responds to iron status in *Saccharomyces cerevisiae*. *J Biol Chem* **277**, 18914-18918.

Yonkovich, J., McKenndry, R., Shi, X. & Zhu, Z. (2002). Copper ion-sensing transcription factor Mac1p post-translationally controls the degradation of its target gene product Ctr1p. *J Biol Chem* **277**, 23981-23984.

Yuan, D. S., Stearman, R., Dancis, A., Dunn, T., Beeler, T. & Klausner, R. D. (1995). The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc Natl Acad Sci U S A* **92**, 2632-2636.

Yuan, D. S., Dancis, A. & Klausner, R. D. (1997). Restriction of copper export in *Saccharomyces cerevisiae* to a late Golgi or post-Golgi compartment in the secretory pathway. *J Biol Chem* **272**, 25787-25793.

Yun, C. W., Ferea, T., Rashford, J., Ardon, O., Brown, P. O., Botstein, D., Kaplan, J. & Philpott, C. C. (2000a). Desferrioxamine-mediated iron uptake in *Saccharomyces cerevisiae*. Evidence for two pathways of iron uptake. *J Biol Chem* **275**, 10709-10715.

Yun, C. W., Tiedeman, J. S., Moore, R. E. & Philpott, C. C. (2000b). Siderophore-iron uptake in saccharomyces cerevisiae. Identification of ferrichrome and fusarinine transporters. *J Biol Chem* **275**, 16354-16359.

Yun, C. W., Bauler, M., Moore, R. E., Klebba, P. E. & Philpott, C. C. (2001). The role of the *FRE* family of plasma membrane reductases in the uptake of siderophore-iron in *Saccharomyces cerevisiae*. *J Biol Chem* **276**, 10218-10223.

Zhou, B. & Gitschier, J. (1997). hCTR1: a human gene for copper uptake identified by complementation in yeast. *Proc Natl Acad Sci U S A* **94**, 7481-7486.

Zhou, H., Cadigan, K. M. & Thiele, D. J. (2003). A copper-regulated transporter required for copper acquisition, pigmentation, and specific stages of development in *Drosophila melanogaster*. *J Biol Chem* **278**, 48210-48218.

Zhou, L. W., Haas, H. & Marzluf, G. A. (1998). Isolation and characterization of a new gene, *sre,* which encodes a GATA-type regulatory protein that controls iron transport in *Neurospora crassa*. *Mol Gen Genet* **259**, 532-540.

Zhou, P. & Thiele, D. J. (1993). Rapid transcriptional autoregulation of a yeast metalloregulatory transcription factor is essential for high-level copper detoxification. *Genes Dev* **7**, 1824-1835.

Zhu, Z., Labbe, S., Pena, M. M. & Thiele, D. J. (1998). Copper differentially regulates the activity and degradation of yeast Mac1 transcription factor. *J Biol Chem* **273**, 1277-1280.

Znaidi, S., Pelletier, B., Mukai, Y. & Labbe, S. (2004). The *Schizosaccharomyces pombe* corepressor Tup11 interacts with the iron-responsive transcription factor Fep1. *J Biol Chem* **279**, 9462-9474.