

## Msn2- and Msn4-Like Transcription Factors Play No Obvious Roles in the Stress Responses of the Fungal Pathogen *Candida albicans*†

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**In *Saccharomyces cerevisiae*, the (C<sub>2</sub>H<sub>2</sub>)<sub>2</sub> zinc finger transcription factors Msn2 and Msn4 play central roles in responses to a range of stresses by activating gene transcription via the stress response element (STRE; CCCCT). The pathogen *Candida albicans* displays stress responses that are thought to help it survive adverse environmental conditions encountered within its human host. However, these responses differ from those in *S. cerevisiae*, and hence we predicted that the roles of Msn2- and Msn4-like proteins might have been functionally reassigned in *C. albicans*. *C. albicans* has two such proteins: CaMsn4 and Mnl1 (for Msn2- and Msn4-like). *CaMSN4*, but not *MNL1*, weakly complemented the inability of an *S. cerevisiae* *msn2 msn4* mutant to activate a STRE-*lacZ* reporter. Also, the disruption of *CaMsn4* and *Mnl1* had no discernible effect upon the resistance of *C. albicans* to heat, osmotic, ethanol, nutrient, oxidative, or heavy-metal stress or upon the stress-activated transcriptome in *C. albicans*. Furthermore, although Cap1-dependent activation of a Yap response element-luciferase reporter was observed, a STRE reporter was not activated in response to stresses in *C. albicans*. Ectopic expression of *CaMsn4* or *Mnl1* did not affect the cellular or molecular responses of *C. albicans* to stress. Under the conditions tested, the putative activation and DNA binding domains of *CaMsn4* did not appear to be functional. These data suggest that *CaMsn4* and *Mnl1* do not contribute significantly to stress responses in *C. albicans*. The data are consistent with the idea that stress signaling in this fungus has diverged significantly from that in budding yeast.**

All living organisms have evolved mechanisms to detect and respond to adverse environmental conditions. In particular, pathogenic microbes must adapt efficiently to stresses imposed by their microenvironments during disease establishment and progression. For example, microbial pathogens must evade or counteract host immune defenses, and they must adapt to changes in pH or nutrient deprivation, depending upon the site of infection.

*Candida albicans* is the major systemic fungal pathogen of humans (6, 44, 45). This fungus is carried as a commensal in the oral and gastrointestinal tracts of many individuals but often causes oral and vaginal infections when fungus-host interactions are disturbed. *C. albicans* also causes systemic infections of internal organs in immunocompromised patients (44), sometimes escaping phagocytic killing, even following engulfment (33). The fact that *C. albicans* is relatively resistant to oxidative stresses (27) might contribute to this. It is likely that *C. albicans* has evolved to counter host defenses in a range of distinct niches within the host, and presumably this is dependent upon specific stress responses.

The relatively benign budding yeast *Saccharomyces cerevisiae* adapts to stress by using several distinct signaling pathways (36). Responses to oxidative and heavy-metal stresses are de-

pendent upon the bZIP transcription factor Yap1 (56), which activates stress-responsive genes via sequences closely related to the Yap response element (YRE; TTA[G/C]TAA) (10). *C. albicans* Cap1, which is a functional homologue of Yap1, mediates responses to oxidative, heavy-metal, and drug-induced stresses (1, 68).

In *S. cerevisiae*, general responses to stresses, including mild heat shock, starvation, osmotic stress, alcohol, and weak acids, are dependent upon the closely related, functionally redundant (C<sub>2</sub>H<sub>2</sub>)<sub>2</sub> zinc finger transcription factors Msn2 and Msn4 (17, 37). There is a third Msn2- and Msn4-like protein in *S. cerevisiae*, Yer130c, but its cellular function remains obscure (<http://db.yeastgenome.org/cgi-bin/SGD>). In response to stresses, Msn2 and Msn4 accumulate in the nucleus (23, 26). This leads to the transcriptional activation of stress-responsive genes via stress response elements (STRE; CCCCT) in their promoters (36, 38, 39). Msn2 and Msn4 appear to interact directly with the STRE element (38), and this interaction is thought to be enhanced by yeast glycogen synthase kinase 3 (25). Msn2- and Msn4-mediated stress responses are down-regulated by the Ras-cyclic AMP pathway (20). Activation of this pathway leads to the phosphorylation of Msn2 by protein kinase A, which causes cytoplasmic accumulation of Msn2 and hence inhibition of the general stress response (23, 24).

Transcript profiling has revealed that *C. albicans* does not display a general stress response under conditions that stimulate such a response in *S. cerevisiae* (15). Hence, our working hypothesis was that the functions of Msn2- and Msn4-like proteins have diverged in *C. albicans*. In this study, we tested this hypothesis by examining Msn2- and Msn4-like proteins in

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TABLE 1. *C. albicans* strains used in this study

Strain	Genotype	Source
SC5314	Wild type	22
CAF2-1	<i>URA3/ura3::λ imm434</i>	19
CA14	<i>ura3::λ imm434/ura3::λ imm434</i>	19
CA18	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG</i>	19
MMY301	<i>ura3::λ imm434/ura3::λ imm434 cap1::hisG/cap1::hisG-URA3-hisG</i>	68
MMC4	<i>ura3::λ imm434/ura3::λ imm434 nrg1::hisG/nrg1::hisG</i>	41
MSC1	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG MNL1/mnl1::hisG-URA3-hisG</i>	This study
MSC2	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG MNL1/mnl1::hisG</i>	This study
MSC3	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG-URA3-hisG/mnl1::hisG</i>	This study
MSC4	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG</i>	This study
MSC5	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG MSN4/msn4::hisG-URA3-hisG</i>	This study
MSC6	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG MSN4/msn4::hisG</i>	This study
MSC7	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG msn4::hisG-URA3-hisG/msn4::hisG</i>	This study
MSC8	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG msn4::hisG/msn4::hisG</i>	This study
MSC9	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG MSN4/msn4::hisG-URA3-hisG</i>	This study
MSC10	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG MSN4/msn4::hisG</i>	This study
MSC11	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG msn4::hisG-URA3-hisG/msn4::hisG</i>	This study
MSC12	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG</i>	This study
MSC13	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG-ADE2 mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG pCRW3 (ADE2) Clp10 (URA3)</i>	This study
MSC14	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG-ADE2 mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG pCRW3 (ADE2) pMNL1 (URA3)</i>	This study
MSC15	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG-ADE2 mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG pCRW3 (ADE2) pMSN4 (URA3)</i>	This study
MSC16	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCRW3 (ADE2) pACT1 (URA3)</i>	This study
MSC17	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCRW3 (ADE2), pACT1-MNL1 (URA3)</i>	This study
MSC18	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCRW3 (ADE2), pACT1-MSN4 (URA3)</i>	This study
SNC7	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG/mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG NRG1/nrg1::hisG-URA3-hisG</i>	This study
SNC8	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG NRG1/nrg1::hisG</i>	This study
SNC9	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG nrg1::hisG-URA3-hisG/nrg1::hisG</i>	This study
SNC10	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG mnl1::hisG/mnl1::hisG msn4::hisG/msn4::hisG nrg1::hisG/nrg1::hisG</i>	This study
SNC11	<i>ura3::λ imm434/ura3::λ imm434 pMET3-VP16 (URA3)</i>	This study
SNC12	<i>ura3::λ imm434/ura3::λ imm434 pMET3-VP16-MNL1<sub>DBD</sub> (URA3)</i>	This study
SNC13	<i>ura3::λ imm434/ura3::λ imm434 pMET3-VP16-MSN4<sub>DBD</sub> (URA3)</i>	This study
CRC116	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCR-lacZ (ADE2) Clp-LexA (URA3)</i>	53
CRC110	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCR-OPlacZ (ADE2) Clp-LexA (URA3)</i>	53
CRC122	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCR-lacZ (ADE2) Clp-LexA-GCN4 (URA3)</i>	53
CRC121	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCR-OPlacZ (ADE2) Clp-LexA-GCN4 (URA3)</i>	53
SNC14	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCR-lacZ (ADE2) Clp-LexA-MSN4 (URA3)</i>	This study
SNC15	<i>ura3::λ imm434/ura3::λ imm434 ade2::hisG/ade2::hisG pCR-OPlacZ (ADE2) Clp-LexA-MSN4 (URA3)</i>	This study

*C. albicans* using a range of approaches, including reverse genetics and genomics. We show that in contrast to *S. cerevisiae*, *C. albicans* Msn2- and Msn4-like proteins do not play significant roles in responses to heat, osmotic, ethanol, or nutrient stress. This indicates that the functions of Msn2- and Msn4-like proteins have been lost in *C. albicans* or that they play differing roles in these yeasts. Our data reinforce the notion that stress responses in *C. albicans* and *S. cerevisiae* have diverged significantly.

#### MATERIALS AND METHODS

**Strains and growth conditions.** The *S. cerevisiae* strains used were W303-1A (*MATa SUC2 ade2 can1 his3 leu2 trp1 ura3* [62]) and the congenic strain Wmsn2msn4 (*MATa SUC2 ade2 can1 his3 leu2 trp1 ura3 msn2::HIS3 msn4::TRP1*) (a generous gift from J. Thevelein). *C. albicans* strains are listed in Table 1. The strains were grown in yeast-peptone-dextrose (YPD) medium (57), yeast-peptone medium containing 3% raffinose or 3% galactose, YPD medium containing 10% fetal calf serum (61), synthetic complete (SC) medium (29), or SD minimal medium (57). Stress phenotypes were assayed by plating 10-fold dilutions of *C. albicans* strains under the conditions specified.

**Strain construction.** The *CaMSN4* and *MNL1* loci were disrupted by Ura blasting, as described previously (19), to generate homozygous single mutants (MSC4 and MSC8) (Table 1). The *msn4::hisG-URA3-hisG* disruption cassette deleted codons 2 to 903 of the 906-codon *CaMSN4* open reading frame (ORF), and the *mnl1::hisG-URA3-hisG* disruption cassette deleted codons 3 to 757 of the 759-codon *MNL1* ORF. The homozygous *mnl1/mnl1 msn4/msn4* double mutant (MSC12) was created by disrupting the *CaMSN4* locus in the *mnl1/mnl1* mutant.

The *CaMSN4* and *MNL1* loci were PCR amplified and cloned into a *URA3*-containing integrating plasmid, Clp10 (40), to create pMSN4 and pMNL1. These plasmids and the control, Clp10, were integrated at the *RPS10* locus in MSC12 to create the strains MSC13 to MSC15 (Table 1). Finally, to generate the homozygous *mnl1/mnl1 msn4/msn4 nrg1/nrg1* triple mutant (SNC10), the *CaNRG1* locus was disrupted in the double mutant (MSC12) using a previously described *nrg1::hisG-URA3-hisG* disruption cassette (41). At each stage of this process, the genotype of each strain was confirmed by both PCR diagnosis and Southern analysis (not shown).

To achieve ectopic expression of *CaMSN4* and *MNL1* in *C. albicans*, these ORFs were PCR amplified using primers MSN4-F, MSN4-R, MNL1-F, and MNL1-R (Table 2); resequenced; and cloned between the *CaACT1* promoter and the *ScCYC1* terminator in pACT1 (63). The resultant plasmids, pACT1, pACT1-MSN4, and pACT1-MNL1, were integrated at the *RPS10* locus in CA18 (40) to create the strains MSC16 to MSC18 (Table 1).

To perform the complementation tests in *S. cerevisiae*, the *CaMSN4* and *MNL1* ORFs from pACT1-MSN4 and pACT1-MNL1 were subcloned into the centromeric plasmid pRS315 (58) under the control of the *S. cerevisiae* *GAL1* promoter. This generated the plasmids pGAL10-MSN4 and pGAL10-MNL1, which were transformed separately into the *S. cerevisiae* *msn2 msn4* mutant alongside the empty pGAL10 control.

To generate the *SalexA-MSN4* fusion, the *CaMSN4* ORF was PCR amplified and cloned into Clp-LexA (53) using the primers MSN4lex-F and MSN4lex-R (Table 2). The forward primer introduced a (Gly)<sub>3</sub>-Pro-(Gly)<sub>2</sub> linker between the amino-terminal LexA domain and the carboxy-terminal CaMsn4 domain (Table 2). The Clp-LexA-MSN4 plasmid was then transformed into *C. albicans* CA18 (Table 1) via integration at the *RPS10* locus, selecting for the plasmid-borne *URA3* marker (53).

To generate a synthetic, codon-optimized VP16 activation domain, oligonu-



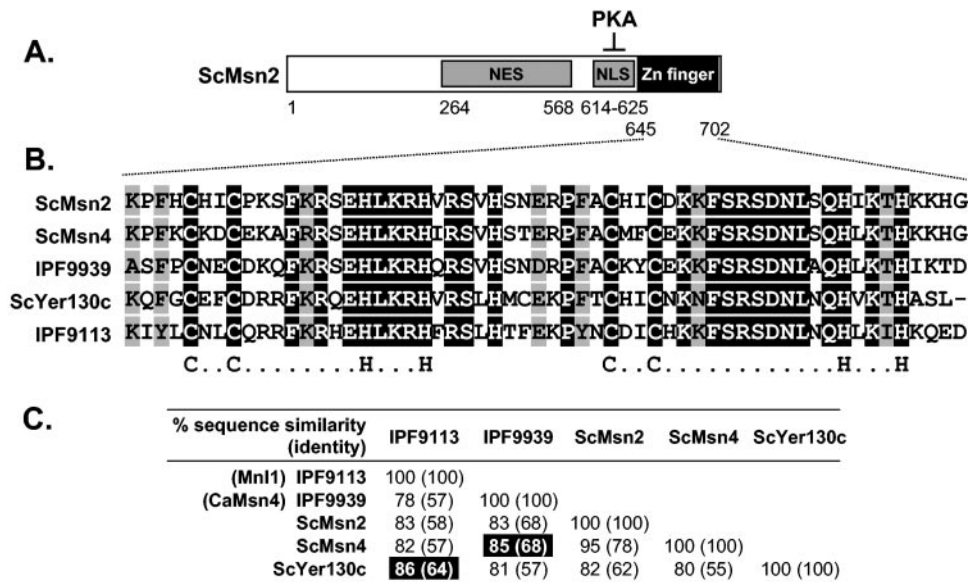


FIG. 1. Comparison of Msn2- and Msn4-like sequences in *C. albicans* and *S. cerevisiae*. (A) Cartoon of *S. cerevisiae* Msn2 (ScMsn2) illustrating the coordinates of the (C<sub>2</sub>H<sub>2</sub>)<sub>2</sub> DNA binding domain (Zn finger), the nuclear export signal (NES), the NLS, and the repression of the NLS by protein kinase A (PKA) (24). (B) Sequence alignments for the (C<sub>2</sub>H<sub>2</sub>)<sub>2</sub> regions of the Msn2- and Msn4-like proteins in *C. albicans* and *S. cerevisiae*. Residues conserved in all proteins, black; conservative substitutions, grey. (C) Percentage sequence similarities (identities) in these regions. IPF9113 and IPF9939 are most similar to the *S. cerevisiae* proteins ScYer130c and ScMsn4, respectively.

cleotides VP16-1T to VP16-4B were sequentially annealed and ligated together (Table 2). Codon optimization was performed using published tables of preferred *C. albicans* codons (5). The full-length VP16 fragment was then PCR amplified using primers VP16-T and VP16-B; the product was cloned into pGEM-T EASY (Promega, Southampton, United Kingdom) and sequenced to create pGEM-VP16. The *S. cerevisiae* CYC1 transcriptional terminator was then cloned downstream of the VP16 domain by inserting the annealed oligonucleotides CYC1-T and CYC1-B into the XhoI site of pGEM-VP16. The resultant BglII-KpnI VP16-CYC1t fragment was subcloned between the BamHI and KpnI sites of the MET3 expression vector, pCaEXPa (7). A new linker was then inserted between the VP16 and CYC1t sequences to introduce additional restriction sites (oligonucleotides Linker-T and Linker-B [Table 2]), and the simian virus 40 (SV40) T-antigen nuclear localization signal (NLS) was inserted between the new NheI and KpnI sites (oligonucleotides NLS-T and NLS-B [Table 2]). This generated the control VP16 expression plasmid, pMET3-VP16 (see Fig. 9).

To express VP16-CaMsn4<sub>DBD</sub> and VP16-Mn1<sub>DBD</sub> fusions in *C. albicans* (see Fig. 9), the zinc finger regions of *MSN4* and *MNL1* were PCR amplified (primers MSNDBD-F, MSNDBD-R, MNLDBD-F, and MNLDBD-R [Table 2]) and cloned between the KpnI and BamHI sites of pMET3-VP16. The resultant plasmids were transformed into *C. albicans* CAI8 (Table 1) via integration at *RPS10*, selecting for the *URA3* marker (40).

**Transcript profiling.** Transcript profiling of the congenic *C. albicans* strains CAI8, MSC12, MSC16, MSC17, and MSC18 was performed on cells growing exponentially in YPD medium. The cells were exposed to the appropriate stress (mild heat shock [23 to 37°C], osmotic stress [0.3 M NaCl], or oxidative stress [0.4 mM H<sub>2</sub>O<sub>2</sub>]) and analyzed 0, 10, 30, and 60 min thereafter. At each time point, MSC12 was compared to its control (CAI8), and MSC17 and MSC18 were compared to their control (MSC16).

Transcript profiling was performed as described previously (15, 43). Briefly, RNA was isolated, Cy3 and Cy5-labeled cDNAs were prepared, and the probes were hybridized with arrays comprising ~95% of *C. albicans* ORFs (43). Slides were scanned at 10- $\mu$ m resolution with a ScanArray 5000 scanner (version 2.11; Packard Bioscience) and quantified using QuantArray software (version 2.0; Packard Bioscience), and data normalization and analysis were performed using GeneSpring software (Silicon Genetics, Redwood City, Calif.) and significance analysis of microarrays (64). Data from at least three independent experiments were used in the analysis. The data are accessible at <http://www.cbr.nrc.ca/genetics/stress/>.

**Sequence analyses.** DNA sequences were analyzed using CandidaDB (<http://genolist.pasteur.fr/CandidaDB>) and the Stanford Genome Database (<http://genome-www.stanford.edu/>).

**Southern, Northern, and Western analyses.** Published methods were used for RNA and DNA preparation, Southern blotting, and Northern analysis (41). Western blotting was performed as described previously (11).

**Reporter assays.** *Renilla reniformis* LUC constructs were made using the basal *ADHI<sub>b</sub>*-RLUC reporter (63), created by introducing a basal *CaADHI* promoter into a derivative of pCRW3 (60). *STRE*-RLUC and *YRE*-RLUC reporters were made by cloning *STRE*- and *YRE*-containing oligonucleotides between BstEII and SpeI sites upstream of the basal promoter (Table 2). pADHI<sub>b</sub>-RLUC, pSTRE-RLUC, and pYRE-RLUC were transformed into *C. albicans* CAI8, and luciferase assays were performed in quadruplicate on independent transformants as described previously (63). An equivalent *STRE-lacZ* fusion was created by cloning a *STRE*-containing oligonucleotide upstream of the basal *S. cerevisiae* *CYC1* promoter (46) and *StlacZ* reporter (65) in Cip10 (40). The activities of SaLexA fusion proteins in *C. albicans* CAI8 were assayed using a *StlacZ* reporter containing the SaLexA operator, as described previously (53). Triplicate  $\beta$ -galactosidase assays were performed on independent transformants using plate overlays and in liquid media (52).

## RESULTS

***C. albicans* has two MSN2- and MSN4-like loci.** Our first aim was to identify *MSN2*- and *MSN4*-like loci in *C. albicans*. Detailed searches of the genome sequence (<http://www-sequence.stanford.edu/group/candida>) and the *C. albicans* genome database (<http://genolist.pasteur.fr/CandidaDB>) revealed two loci with significant sequence similarity to *S. cerevisiae* *MSN2*, *MSN4*, or *YER130c*: IPF9113 (orf19.6121) and IPF9939 (orf19.4752). These *C. albicans* proteins display significant sequence similarity in their putative DNA binding domains to each other, as well as to *S. cerevisiae* Msn2, Msn4, and Yer130c (Fig. 1). The sequence similarities between the *C. albicans* and *S. cerevisiae* proteins were limited to the putative DNA binding domains. This is also the case for other functionally related

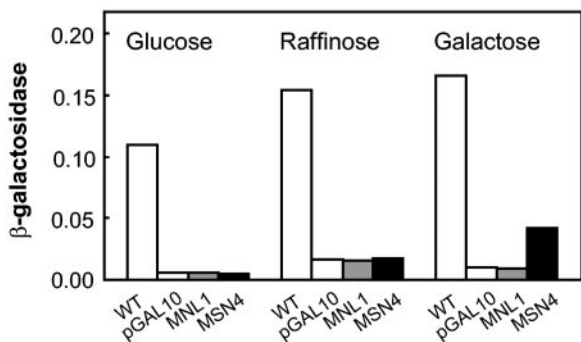


FIG. 2. Ability of *CaMSN4* and *MNL1* to complement an *S. cerevisiae* *msn2 msn4* double mutation. *CaMSN4* and *MNL1* were expressed using the *ScGAL1* promoter in *S. cerevisiae* strain Wmsn2msn4 containing a *STRE-lacZ* reporter. Cells were grown to mid-exponential phase on glucose, raffinose, or galactose and then exposed to 0.3 M NaCl for 30 min, and  $\beta$ -galactosidase activities were measured (Miller units). *S. cerevisiae* W303-1A (WT); *S. cerevisiae* Wmsn2msn4 containing the empty expression vector pGAL10 (pGAL10); pGAL10-MNL1 (MNL1), or; pGAL10-MSN4 (MSN4). Errors were <10%, and similar data were obtained in three independent experiments.

transcription factors in these fungi, such as Gcn4, Nrg1, and Rox1/Rfg1 (2, 28, 41, 63).

IPF9113 is related to Msn2 but is most similar to Yer130c. However, *C. albicans* genes should not be named using the formal gene names of *S. cerevisiae* orthologues. Hence, IPF9113 was provisionally named *MNL1* (for Msn2- and Msn4-like protein). IPF9939 is most similar to Msn4 and therefore was provisionally named *CaMsn4*. To avoid confusion, we use the prefixes “Ca” and “Sc” to distinguish *C. albicans* and *S. cerevisiae* orthologues.

**CaMSN4 weakly complements an *S. cerevisiae* *msn2 msn4* double mutation.** The next objective was to test whether *CaMsn4* and *Mnl1* are functional homologues of *ScMsn2*, *ScMsn4*, or *ScYer130c*. Unfortunately, *S. cerevisiae* *yer130c*

mutants display no obvious phenotype (<http://db.yeastgenome.org/cgi-bin/SGD>), and therefore it was not possible to perform a complementation test in such strains. However, we were able to exploit the transcriptional defect of *S. cerevisiae* *msn2 msn4* cells with respect to *STRE-lacZ* activation (38).

*GAL1-MSN4* and *GAL1-MNL1* fusions were constructed to drive the expression of these *C. albicans* ORFs in *S. cerevisiae* using the *ScGAL1* promoter. These centromeric plasmids, and the empty pGAL10 control, were transformed into *S. cerevisiae* wild-type and *msn2 msn4* cells. These cells were exposed to osmotic stress during exponential growth on glucose (to repress the *ScGAL1* promoter), raffinose (to derepress the *ScGAL1* promoter), or galactose (to activate the *ScGAL1* promoter). As expected (38), wild-type *S. cerevisiae* cells displayed *STRE-lacZ* induction in response to the stress, and the *S. cerevisiae* *msn2 msn4* cells containing the empty pGAL10 plasmid showed no significant induction (Fig. 2). The *STRE-lacZ* reporter was not induced in cells containing pGAL10-MNL1, indicating that *MNL1* is unable to complement the double *msn2 msn4* mutation in *S. cerevisiae*. This was consistent with the idea that IPF9113 is most closely related to *S. cerevisiae* Yer130c (Fig. 1). In contrast, cells containing pGAL10-MSN4 displayed weak *STRE-lacZ* activation during growth on galactose. This activation was not apparent during growth on raffinose. This suggested that *CaMSN4* is able to complement the double *msn2 msn4* mutation, but only weakly.

**Cellular phenotypes of *C. albicans* *msn4* and *mnl1* mutants.** Isogenic homozygous null mutants were generated to examine the roles of *CaMsn4* and *Mnl1* in *C. albicans* (Table 1). This involved the sequential deletion of both alleles for each locus in this diploid fungus using standard Ura-blasting procedures (19). Each *CaMSN4* allele was inactivated by deleting essentially all of the 759-codon ORF and inserting the Ura blaster cassette (see Materials and Methods). Similarly, *MNL1* was disrupted by replacing all of the 906-codon ORF with the Ura blaster cassette. A double mutant was then created by disrupt-

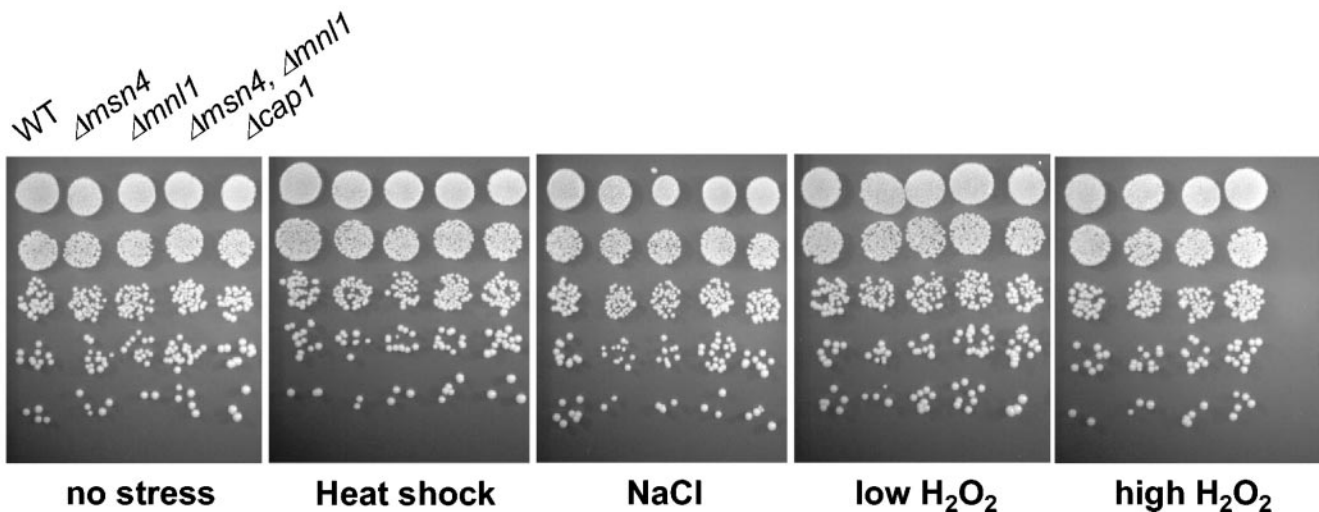


FIG. 3. Inactivation of *CaMsn4* and *Mnl1* does not alter stress resistance in *C. albicans*. *C. albicans* strains were exposed to a wide range of stresses, including no stress (YPD; 30°C), mild heat shock (25 to 37°C), 1.0 M NaCl, 0.4 mM H<sub>2</sub>O<sub>2</sub>, and 2.5 mM H<sub>2</sub>O<sub>2</sub>. Strains: CAI8 (WT), MSC8 ( $\Delta$ *msn4*), MSC4 ( $\Delta$ *mnl1*), MSC12 ( $\Delta$ *msn4*,  $\Delta$ *mnl1*), and MMY301 ( $\Delta$ *cap1*) (Table 1). Where necessary, strains were transformed with *CIP10* (*URA3*) and *pCRW3* (*ADE2*) to make them prototrophic.

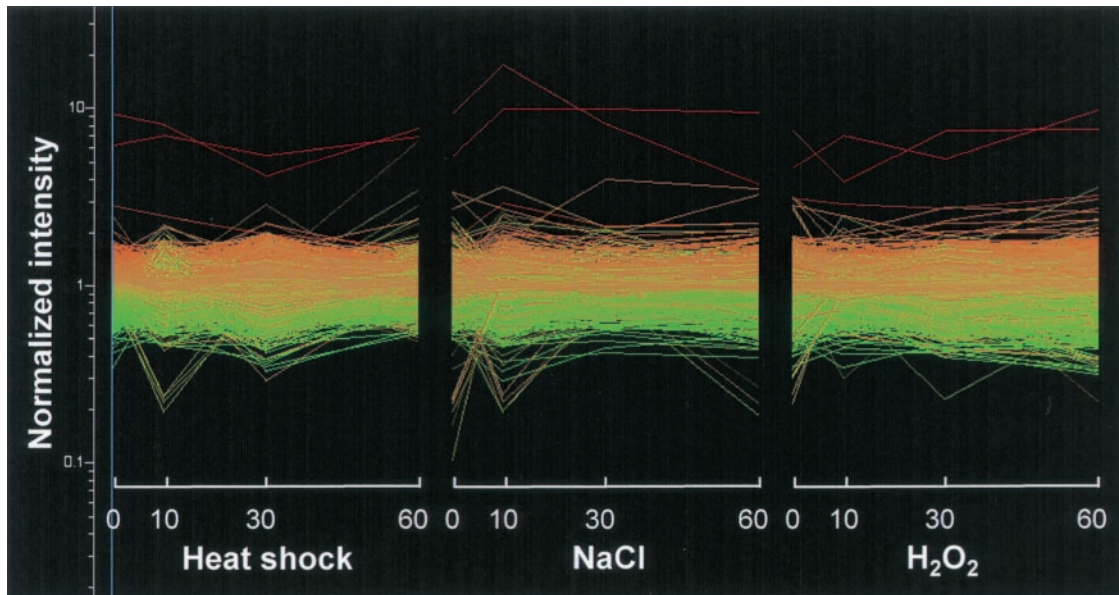


FIG. 4. Inactivation of CaMsn4 and Mnl1 does not significantly affect the stress transcriptomes of *C. albicans*. Strains CAI8 (wild type) and MSC12 ( $\Delta msn4\Delta mnl1$ ) were transformed with CIP10 (*URA3*) and pCRW3 (*ADE2*) to make them prototrophic. The cells were exposed to a mild heat shock (25 to 37°C), an osmotic stress (0.3 M NaCl), or an oxidative stress (0.4 mM H<sub>2</sub>O<sub>2</sub>); RNA was isolated after 0, 10, 30, and 60 min; and transcript profiling was performed (15). Mean ratios ( $\Delta msn4 \Delta mnl1$  mutant versus wild type) of the normalized signal intensities for each gene under each experimental condition were calculated using data from three independent experiments. Each line represents the effect of the  $\Delta msn4 \Delta mnl1$  double mutation upon the expression of a gene under the conditions shown; a ratio of 1.0 indicates that any gene regulation observed in wild-type cells is not affected by the inactivation of CaMsn4 and Mnl1.

ing the *CaMSN4* locus in the homozygous *mnl1/mnl1* null mutant (Table 1). The loss of *CaMSN4* and *MNL1* mRNAs in the corresponding mutants was confirmed by Northern analysis (not shown).

An *S. cerevisiae* *msn2 msn4* double mutant displays sensitivity to general stresses (38), but *S. cerevisiae* *yer130c* mutants display no obvious phenotype. If *CaMSN4* and *MNL1* have been functionally reassigned in *C. albicans*, the corresponding mutants might not be expected to display stress phenotypes. Hence, we examined the sensitivities of the *C. albicans* *msn4/msn4* mutants, the *mnl1/mnl1* mutants, and double mutants under a wide range of stress conditions. These included mild heat shock (25 to 37°C), osmotic stress (0.3 to 2 M NaCl), oxidative stress (0.4 to 2.5 mM H<sub>2</sub>O<sub>2</sub>) (Fig. 3), heavy-metal stress (0.1 to 1.0 mM CdSO<sub>4</sub>), ethanol stress (7%), and carbon starvation (growth to stationary phase and on minimal medium lacking glucose and containing glycerol) (not shown). As predicted, neither the *msn4/msn4* and *mnl1/mnl1* single mutants nor the double *msn4/msn4 mnl1/mnl1* mutant displayed any obvious phenotype under any of these conditions. As a control, we examined the behavior of a *cap1/cap1* mutant. As expected (1), this mutant displayed sensitivity to H<sub>2</sub>O<sub>2</sub> but not to the other stresses tested (Fig. 3).

The observation that *C. albicans* *msn4/msn4 mnl1/mnl1* mutants lacked an obvious stress phenotype was consistent with our working hypothesis that the functions of CaMsn4 and Mnl1 have been reassigned in *C. albicans*. However, the data did not exclude the possibility that these factors play a nonessential role in stress responses in this fungus. It remained a formal possibility that functional redundancy might exist between CaMsn4/Mnl1 and some other unidentified factor(s) in

*C. albicans*. We reasoned that some hidden role in stress responses might be revealed by ectopic expression of CaMsn4 or Mnl1. Hence, the *C. albicans* *ACT1* promoter was used to drive ectopic expression of *CaMSN4* and *MNL1*. This promoter has been used successfully to drive ectopic expression of other transcription factors in *C. albicans*. For example, *ACT1-CaNRG1* and *ACT1-CaGCN4* fusions have been shown to confer morphological and metabolic phenotypes upon *C. albicans* (2, 63). However, *C. albicans* strains carrying *ACT1-CaMSN4* or *ACT1-MNL1* fusions displayed no significant elevation in resistance to heat shock, osmotic stress, heavy-metal stress, or oxidative stress (not shown). This was consistent with the idea that CaMsn4 and Mnl1 play no significant roles in the stress responses examined.

**Molecular phenotypes of *C. albicans* *msn4* and *mnl1* mutants.** It was possible that by examining the cellular phenotypes of *msn4* and *mnl1* mutants we might have missed subtle influences of CaMsn4 and Mnl1 upon *C. albicans* stress responses. However, we reasoned that subtle roles for these proteins would be revealed by analyzing the molecular phenotypes of the corresponding null mutants. Hence, transcript profiling was used to compare the double *msn4/msn4 mnl1/mnl1* mutant with its isogenic wild-type parent during exposure to a mild heat shock (23 to 37°C), an osmotic shock (0.3 M NaCl), or an oxidative stress (0.4 mM H<sub>2</sub>O<sub>2</sub>). These conditions were chosen because they had been shown previously to generate specific stress responses in the *C. albicans* transcriptome (15). The strains were compared at 0, 10, 30, and 60 min after exposure to each stress. A high degree of statistical reproducibility was observed for the three independent experiments that were performed for each time point. However, inactivation of

TABLE 3. Effects of inactivating CaMsn4 and Mnl1 upon gene induction in response to osmotic stress

Gene	Expression ratio								Function <sup>d</sup>
	WT stress/WT no stress <sup>a,b</sup> at time (min):				<i>msn4 mnl1</i> stress/WT stress <sup>c</sup> at time (min):				
	0	10	30	60	0	10	30	60	
<i>orf19.7284</i>	0.9	20.2	55.3	1.4	0.8	1.1	0.8	0.7	Unknown function
<i>orf19.5302</i>	0.9	14.8	27.8	4.4	1.1	2.0	1.3	0.8	Unknown function
<i>MSC1</i>	1.0	14.1	23.2	1.7	0.8	0.9	0.9	0.7	Meiotic sister chromatid recombination
<i>orf19.5070</i>	0.9	13.7	1.3	1.1	1.2	1.1	0.9	1.1	Unknown function
<i>CTA1</i>	1.0	11.8	7.7	1.2	1.0	1.0	1.1	0.8	Peroxisomal catalase A
<i>orf19.2048</i>	0.9	10.8	3.2	0.7	1.5	1.1	1.1	1.1	Unknown function
<i>DDR48</i>	1.1	9.9	12.2	5.0	1.0	1.1	1.3	1.1	DNA damage-responsive protein
<i>orf19.2344</i>	1.0	9.7	14.2	1.8	1.1	0.8	1.2	0.8	Unknown function
<i>orf19.7350</i>	1.1	9.3	5.1	2.3	0.5	0.5	0.8	0.6	Unknown function
<i>CEX16</i>	0.8	8.8	1.3	2.4	1.4	1.5	1.8	1.5	Putative interaction with heat shock proteins and chaperones
<i>orf19.3932</i>	1.1	8.3	7.5	1.2	1.0	0.9	0.7	0.8	Unknown function
<i>orf19.2737</i>	0.9	8.3	8.0	0.9	1.0	0.9	1.0	1.0	FGGY family of carbohydrate kinases
<i>orf19.7296</i>	0.9	8.1	0.6	0.7	0.8	0.9	1.0	0.6	Unknown function
<i>ADH6</i>	1.1	7.5	6.1	1.0	0.9	0.9	1.0	1.0	Alcohol dehydrogenase
<i>DCW2</i>	1.0	7.1	1.9	1.3	1.1	1.1	1.0	1.0	GP1-anchored protein with cell wall role
<i>SGA1</i>	0.9	7.0	0.9	1.3	1.1	1.2	1.0	1.2	Glucoamylase
<i>orf19.692</i>	0.9	6.1	0.6	0.9	1.1	1.2	1.0	1.0	Unknown function
<i>YBR56</i>	1.0	5.9	6.9	1.0	1.0	0.8	1.0	0.8	Putative exo-1,3-beta-glucanase
<i>AGP2</i>	1.0	5.8	1.7	2.2	1.0	1.0	1.0	1.0	Putative amino acid permease
<i>orf19.3007.2</i>	1.0	5.5	2.7	0.8	1.2	1.0	1.0	0.9	Unknown function
<i>LRR</i>	0.9	5.1	1.1	1.0	0.6	0.8	1.3	0.7	Leucine-rich repeat protein

<sup>a</sup> Data from Enjalbert et al. (15).

<sup>b</sup> The osmotic stress was 0.3 M NaCl. Similar observations were obtained for the heat and oxidative stresses (not shown). WT, wild type.

<sup>c</sup> Data from this study (Fig. 4).

<sup>d</sup> Gene information from <http://candida.bri.nrc.ca/candida/index.cfm> and <http://genolis1.pasteur.fr/CandidaDB/>.

CaMsn4 and Mnl1 had no significant effect upon the transcriptional responses of *C. albicans* to these stresses (Fig. 4). Wild-type *C. albicans* cells display a well-defined transcriptional response to the osmotic, oxidative, and heat stresses examined in this study (15). When the transcript profiles of *msn4/msn4 mnl1/mnl1* and wild-type cells were compared, the expression

ratios for most genes approximated to 1 at each time point under each condition (Fig. 4 and Table 3). This indicates that the inactivation of CaMsn4 and Mnl1 had no significant effect upon the expression of almost all *C. albicans* genes. Hence, all stress-induced transcription was retained in the *msn4/msn4 mnl1/mnl1* cells under the stress conditions examined.

The expression of only one stress-induced gene was consistently reduced more than twofold in the *msn4/msn4 mnl1/mnl1* mutant: *CaYPL088*. Northern analysis confirmed that this transcript was induced moderately by osmotic stress in wild-type cells and that this increase was blocked in *msn4/msn4 mnl1/mnl1* cells (Fig. 5). This gene encodes a protein with sequence similarity to *Agrobacterium tumefaciens* MocA, but the biological significance of this change, if any, is not clear.

Signals for two probes on the microarray were constitutively elevated in *msn4/msn4 mnl1/mnl1* cells. However, both probes corresponded to the same transcript (*CaYIR035*). The elevation in *CaYIR035* mRNA levels in the unstressed double mutant was confirmed by Northern analysis (Fig. 5). Northern analysis of the single *msn4/msn4* and *mnl1/mnl1* mutants suggested that CaMsn4 plays a greater role than Mnl1 in the regulation of *CaYIR035*, which is predicted to encode a short-chain dehydrogenase. Again, the biological significance of this change is not clear.

The *CaMSN4* transcript was constitutively reduced in *msn4/msn4 mnl1/mnl1* cells, confirming our earlier Northern analyses, which indicated that *msn4/msn4* cells lacked *CaMSN4* mRNA (not shown). The transcript profiling data of Enjalbert and coworkers (15) indicate that *CaMSN4* and *MNLI* are expressed under the heat, osmotic, and oxidative stress condi-

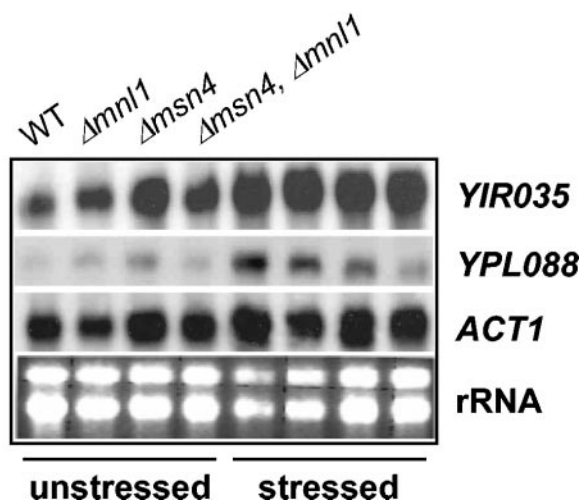


FIG. 5. Confirmation of the responses of the *CaYPL088* and *CaYIR035* transcripts by Northern analysis. Northern blotting was used to compare the levels of the *CaYPL088* and *CaYIR035* transcripts in unstressed *C. albicans* cells and in cells exposed to an osmotic stress (0.3 M NaCl) for 10 min. CA18 (WT), MSC4 ( $\Delta mnl1$ ), MSC8 ( $\Delta msn4$ ), and MSC12 ( $\Delta msn4, \Delta mnl1$ ). The *ACT1* mRNA and rRNAs were used as internal loading controls.

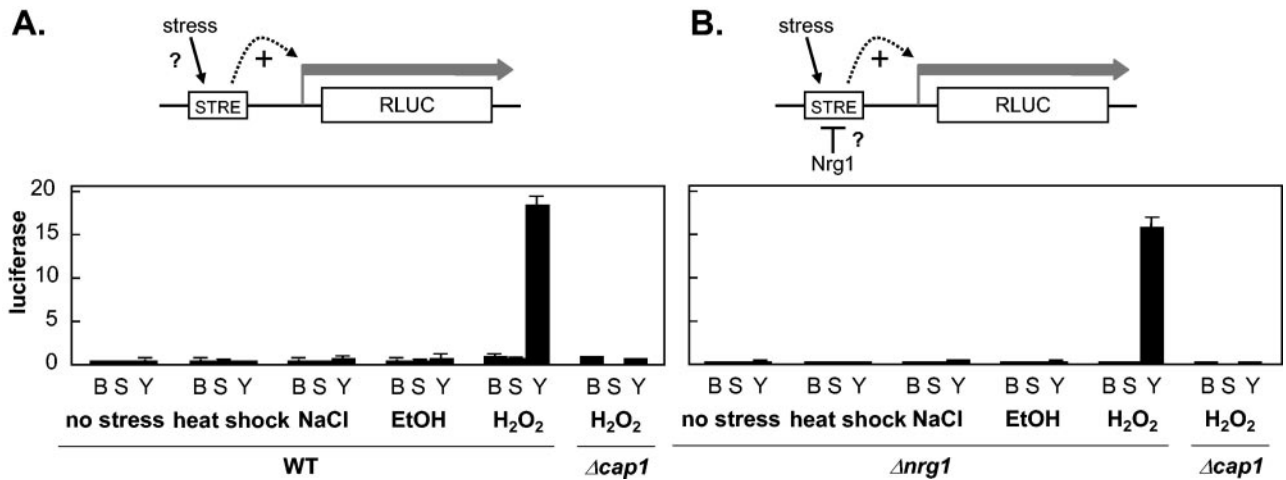


FIG. 6. The STRE does not mediate stress-activated transcription in *C. albicans*. (A) To test the working model that some stresses activate transcription via the STRE in *C. albicans*, the expression of basal RLUC (B), STRE-RLUC (S), and YRE-RLUC (Y) fusions were monitored in CAI8 (WT) following exposure to a range of stresses: no stress (YPD; 30°C), mild heat shock (25 to 37°C), 0.3 M NaCl, 7% ethanol (EtOH), and 2.5 mM  $H_2O_2$ . The effect of Cap1 inactivation upon the response to 2.5 mM  $H_2O_2$  was measured as a control using strain MMY301 (Table 1). Luciferase levels were measured in triplicate for three independent transformants ( $10^5$  relative light units). (B) To test the hypothesis that CaNrg1 might repress STRE activation, the same experiment was performed using strain MMC4 ( $\Delta nrg1$ ) (Table 1).

tions examined and that their transcript levels do not change significantly under these conditions.

Although unlikely, it was possible that the effects of the *msn4/msn4 mnl1/mnl1* mutations upon the transcriptome might have been masked by functional redundancy with some unknown factor. Therefore, we examined the effects of the *ACT1-CaMSN4* and *ACT1-MNL1* fusions upon the *C. albicans* transcriptome. Again, the three independent transcript-profiling experiments performed for each experimental condition were highly reproducible. However, the ectopic expression of CaMsn4 or Mnl1 had no significant effect upon the transcript profile relative to the pACT1 control, even following exposure of the *C. albicans* cells to heat, osmotic, or oxidative stress (not

shown). Therefore, transcript profiling revealed no obvious roles for CaMsn4 or Mnl1 during the *C. albicans* stress responses tested or during exponential growth on glucose. This was consistent with our working hypothesis.

**The STRE in *C. albicans*.** *S. cerevisiae* Msn2 and Msn4 activate transcription via the STRE (38), and the role of the STRE in mediating transcriptional responses to stress is conserved in other fungi (47). Many *C. albicans* genes that respond to stress contain STRE-like sequences in their promoters. Furthermore, we have shown that other *S. cerevisiae* regulatory elements, such as GCRE and YRE, are conserved in *C. albicans* (34, 63). However, if CaMsn4 and Mnl1 have been functionally reassigned, we predicted that STRE-like sequences might not

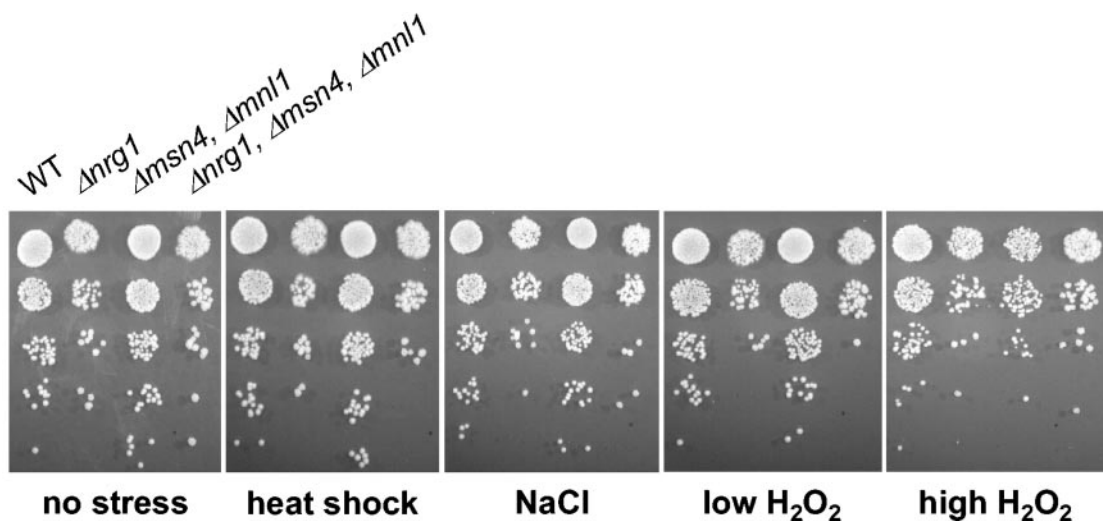


FIG. 7. CaNrg1, CaMsn4, and Mnl1 do not display synthetic stress phenotypes. *C. albicans* cells were exposed to a wide range of stresses, including no stress (YPD; 30°C), mild heat shock (25 to 37°C), 1.0 M NaCl, 0.4 mM  $H_2O_2$ , and 2.5 mM  $H_2O_2$ . Strains: CAI8 (WT), MMC4 ( $\Delta nrg1$ ), MSC12 ( $\Delta msn4, \Delta mnl1$ ), and SNC10 ( $\Delta nrg1, \Delta msn4, \Delta mnl1$ ) (Table 1). The strains were transformed with CIP10 (*URA3*) and/or pCRW3 (*ADE2*) to make them prototrophic.



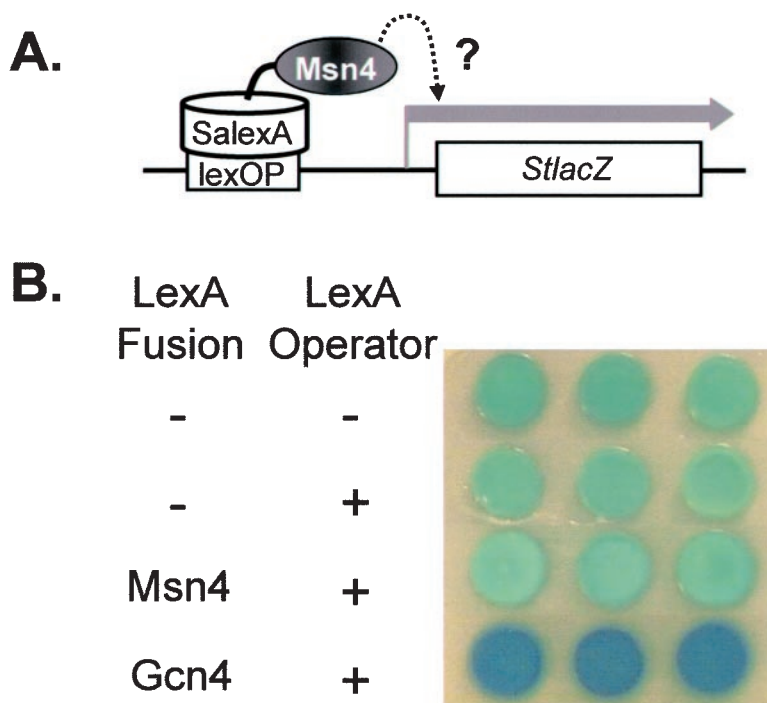


FIG. 8. Transcriptional activity of the putative activation domain of CaMsn4. The activity of a SaLexA-Msn4 fusion protein was compared to that of a SaLexA-Gcn4 fusion (positive control) in *C. albicans* using a *StlacZ* reporter carrying the SaLexA operator. (A) Cartoon illustrating the experimental rationale. (B)  $\beta$ -Galactosidase activities were assayed in strains CRC116, CRC110, CRC121, and SNC15 (Table 1) with three independent transformants using an X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) overlay assay developed for 1 h. -, absent; +, present.

mediate transcriptional responses to general stresses in *C. albicans*.

To test this, we introduced four tandem STRE upstream of the basal *C. albicans* reporter, *ADH1<sub>b</sub>-RLUC* (63), to create *STRE-RLUC*. As a positive control, we constructed an analogous *YRE-RLUC* reporter containing four tandem YRE. As expected, the *YRE-RLUC* reporter did not respond to a mild heat shock, 0.3 M NaCl, or 7% ethanol but was activated in response to 2.5 mM H<sub>2</sub>O<sub>2</sub> in a Cap1-dependent fashion (Fig. 6A). In contrast, the STRE reporter displayed no significant activation above basal levels following exposure to these heat, osmotic, ethanol, or oxidative stresses.

It was possible that, although the *YRE-RLUC* reporter had responded appropriately to an oxidative stress, something about the design of the *STRE-RLUC* reporter had inhibited STRE-mediated transcriptional activation. Therefore, we generated a second reporter in which the STRE were provided with alternative flanking nucleotides, an alternative basal promoter region (*ScCYC1*), and an alternative reporter (*StlacZ*) (65). However, no STRE-mediated transcriptional activation was observed using this alternative reporter (not shown). Therefore, no obvious role was observed for the STRE with respect to stress-mediated transcription in *C. albicans*. This was consistent with the lack of involvement of CaMsn4 and Mnl1 in general stress responses.

**Potential overlap between CaMsn4, Mnl1, and CaNrg1 regulons in *C. albicans*.** Previously, CaNrg1 was identified as a transcriptional repressor that mediates its effects in *C. albicans* via the Nrg1 response element (NRE) (41, 42). The consensus

sequence for the NRE [(A/C)(A/C/G)C<sub>3</sub>T] (41) is closely related to the STRE (CCCCT), so that STREs appear to be a subset of potential NREs. Therefore, in principle, CaNrg1 might repress STRE-mediated transcriptional activation in *C. albicans*. It follows, therefore, that there might be some overlap between CaMsn4, Mnl1, and CaNrg1 regulons in *C. albicans* and that this overlap might have masked the activities of CaMsn4, Mnl1, and STRE in the above-mentioned experiments. A prediction of this working hypothesis was that these activities would be unmasked by inactivating CaNrg1.

To test this, we generated a *C. albicans msn4/msn4 mnl1/mnl1 nrg1/nrg1* triple mutant and compared its phenotype to those of wild-type, *nrg1/nrg1*, and *msn4/msn4 mnl1/mnl1* cells (Fig. 7). Inactivation of CaNrg1 derepresses filamentous growth in *C. albicans* (2, 41), and therefore, cells carrying the *nrg1/nrg1* mutation formed wrinkly colonies. However, the triple mutant displayed no significant difference from *nrg1/nrg1* cells with respect to stress sensitivity (Fig. 7). Again, this reinforced the idea that CaMsn4 and Mnl1 do not have significant roles in the *C. albicans* stress responses examined, even in the absence of CaNrg1.

Does CaNrg1 repress STRE-mediated transcriptional activation in response to stresses? This was tested by assaying the activity of the *STRE-RLUC* reporter in *nrg1/nrg1* cells (Fig. 6B). The inactivation of CaNrg1 did not release any significant activation of the reporter following exposure to mild heat, osmotic, ethanol, or oxidative stress. Therefore, no significant overlap between the CaMsn4, Mnl1, and CaNrg1 regulons was observed in this study.

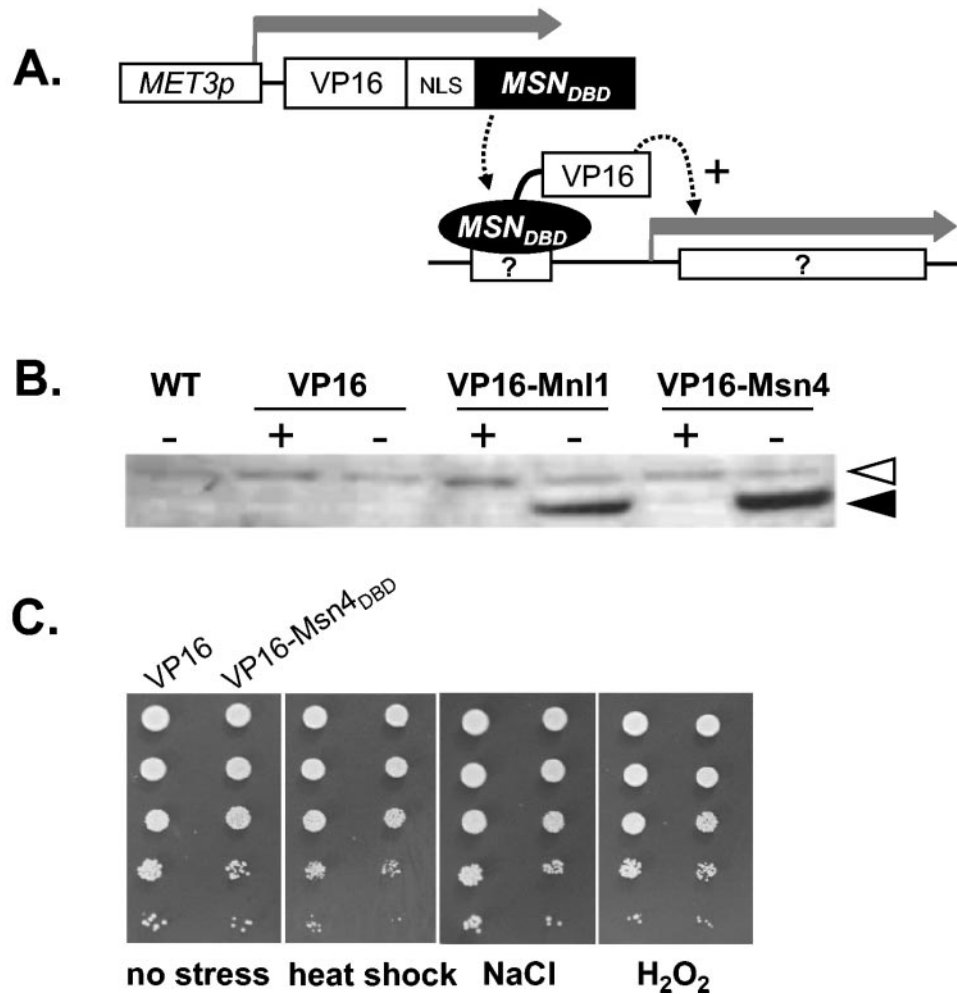


FIG. 9. Expression of a VP16-CaMsn4<sub>DBD</sub> fusion in *C. albicans* causes no obvious stress phenotype. (A) Cartoon illustrating the experimental rationale to identify gene targets of CaMsn4. The *CaMET3* promoter is repressed by methionine and cysteine (7). (B) Western blot with anti-VP16 antibody showing regulated expression of *MET3-VP16-MSN4<sub>DBD</sub>* in *C. albicans*. Wild type, CAI4 (WT; Table 1); SNC11 (VP16); SNC12 (VP16-Mnl1); SNC13 (VP16-Msn4); no methionine or cysteine (-); 10 mM methionine and 10 mM cysteine (+); open arrow, nonspecific band; closed arrow, VP16 fusion protein. (C) Phenotype of *C. albicans* CAI4 cells expressing VP16-CaMsn4<sub>DBD</sub> or the VP16 control on plates lacking methionine and cysteine. No stress (SC; 30°C), mild heat shock (25 to 37°C), 1.0 M NaCl, and 2.5 mM H<sub>2</sub>O<sub>2</sub>.

**Functionality of CaMsn4 in *C. albicans*.** Northern analysis and transcript profiling had indicated that the *CaMSN4* gene is expressed, and the complementation experiment suggested that CaMsn4 might have some transcriptional activity at least in *S. cerevisiae* (Fig. 2). However, our cellular and molecular analyses had revealed no obvious function for this protein in *C. albicans*. Therefore, we assayed the activities of the putative transcriptional activation and DNA binding domains of CaMsn4 in *C. albicans*.

LexA fusions have been used to examine the activities of specific transcription factors in *S. cerevisiae* (3, 30). Hence, we expressed CaMsn4 as a LexA fusion protein in *C. albicans* and asked whether it could activate the expression of a reporter gene carrying the corresponding *lexA* operator in its promoter (Fig. 8A). *Staphylococcus aureus lexA* was used because it lacks CUG codons (53), which are decoded as serine, not leucine, in *C. albicans* (55). Hence, a *SalexA-CaMSN4* fusion was introduced into a *C. albicans* strain containing a *Streptococcus ther-*

*mophilus lacZ* reporter gene under the control of a *SalexA* operator sequence (53). Control strains contained a *StlacZ* reporter lacking the *SalexA* operator, and these generated basal levels of  $\beta$ -galactosidase in *C. albicans* (Fig. 8B). These levels were not affected significantly by the introduction of the *S. aureus lexA* operator. As expected (53), a control LexA-Gcn4 fusion showed significant *StlacZ* activation. However, the LexA-CaMsn4 fusion displayed no significant activation, even following exposure to osmotic (1.0 M NaCl) or ethanol (7%) stress. Therefore, we were unable to detect any significant transcriptional activation by CaMsn4.

To examine the functionality of the zinc finger domain of CaMsn4, codons 554 to 759 of *CaMSN4* were fused to a synthetic codon-optimized VP16 transcriptional activation domain (see Materials and Methods). This VP16 domain has been shown to activate transcription in *S. cerevisiae* (12, 50). The VP16-CaMsn4<sub>DBD</sub> fusion was expressed in *C. albicans* using the methionine-conditional *MET3* promoter (7) and tar-

geted to the nucleus using a synthetic SV40 T-antigen nuclear localization signal (Fig. 9A). Control cells contained the empty expression plasmid pMET3-VP16 or pMET3-VP16-MNL1. Methionine-conditional expression of the VP16-CaMsn4<sub>DBD</sub> fusion and the control VP16-Mnl1<sub>DBD</sub> protein in *C. albicans* was confirmed by Western blotting (Fig. 9B). However, no obvious growth or stress phenotype was observed for cells expressing the VP16-CaMsn4<sub>DBD</sub> fusion compared with control cells (Fig. 9C).

We reasoned that *C. albicans* genes containing the CaMsn4 DNA binding site would be activated artificially following expression of this VP16-CaMsn4<sub>DBD</sub> fusion. The intention was to identify these CaMsn4 target genes, and hence the CaMsn4 DNA binding site. Therefore, transcript profiling was performed to compare the molecular responses of *C. albicans* cells to the VP16-CaMsn4<sub>DBD</sub> fusion and the VP16 control. Highly reproducible signals were obtained for four independent hybridizations, but no significant differences were observed between the transcript profiles of pMET3-VP16-CaMSN4<sub>DBD</sub> and pMET3-VP16 cells. This reinforced the idea that CaMsn4 no longer retains activity as a transcriptional activator in *C. albicans*.

## DISCUSSION

In general, there appears to be a high degree of conservation between *S. cerevisiae* and *C. albicans* with respect to their signal transduction modules. These include the MAP kinase module involved in mating responses, the adenylyl cyclase-protein kinase A module that regulates cellular morphogenesis, the Rim101 module involved in pH signaling, the Gcn4 module that activates general amino acid control, the Yap1 module that mediates oxidative stress response, and the Hog1 module involved in osmotic stress responses (1, 4, 13, 14, 16, 18, 31, 32, 35, 48, 51, 54, 59, 63, 66, 68). However, transcript profiling has highlighted significant differences between the general stress responses of *C. albicans* and those of *S. cerevisiae* (15). Hence, we anticipated significant differences between the pathogenic fungus and the relatively benign fungus with respect to their Msn2- and Msn4-like signaling modules.

*C. albicans* has two Msn2- and Msn4-like proteins. IPF9939 (orf19.4752) was called *CaMSN4* on the basis that its product is most similar to *S. cerevisiae* Msn4 (Fig. 1), and it was able to complement an *S. cerevisiae* *msn2 msn4* double mutation, albeit weakly (Fig. 2). IPF9113 (orf19.6121), which was most similar to *S. cerevisiae* *YER130c*, was called *MNL1* on the basis of its similarity to Msn2- and Msn4-like proteins (Fig. 1). Furthermore, *MNL1*, like *S. cerevisiae* *YER130c*, was unable to complement an *S. cerevisiae* *msn2 msn4* double mutation (Fig. 2). The existence of a single *C. albicans* orthologue (*CaMSN4*) of the functionally redundant *MSN2-MSN4* gene pair in *S. cerevisiae* is consistent with the idea that, during fungal evolution, genome duplication occurred after the divergence of *C. albicans* and *S. cerevisiae* (67).

To test our working model that CaMsn2- and Msn4-like proteins in *C. albicans* have been functionally reassigned, we examined their roles in detail. As expected, neither CaMsn4 nor Mnl1 appears to play an obvious role in stress responses. This conclusion was based on numerous complementary observations. (i) Inactivation of *CaMSN4* and *MNL1* did not

increase the sensitivity of *C. albicans* to any of the numerous stresses tested (Fig. 3). This differs from the situation in *S. cerevisiae*, where an *msn2 msn4* mutant is more sensitive to general stresses (38). It also contrasts with *CAP1*, the inactivation of which renders *C. albicans* more sensitive to oxidative stresses (Fig. 3) (1). (ii) Inactivation of *CaMSN4* and *MNL1* did not affect the *C. albicans* transcriptome during responses to mild heat shock or osmotic or oxidative stress (Fig. 4 and Table 3). Again, this contrasts with *S. cerevisiae*, in which the inactivation of Msn2 and Msn4 inhibits transcriptional responses to many stresses (8, 21). Subtle effects of CaMsn4 or Mnl1 upon stress responses might have been missed in our analyses of cellular stress responses. However, such effects are unlikely to have been missed by transcript profiling, which is exquisitely sensitive to environmental change (8, 9, 15, 21, 43). (iii) Ectopic expression of *CaMSN4* or *MNL1* did not increase the tolerance of *C. albicans* to stresses and did not affect the *C. albicans* transcriptome significantly during responses to stress (not shown). The *ACT1* promoter has been used successfully to generate overexpression phenotypes for at least two other transcription factors in *C. albicans* (*CaNrg1* and *CaGcn4*) (2, 63). Nevertheless, we are unable to exclude the possibility that the absence of cellular and molecular phenotypes was due to a lack of overexpression, improper folding, or mislocalization of CaMsn4 and Mnl1. (iv) The STRE did not mediate transcriptional activation in response to stresses in *C. albicans* (Fig. 6), although CaMsn4 was capable of activating the transcription of a STRE reporter in *S. cerevisiae* in a stress-dependent fashion, albeit weakly (Fig. 2). Therefore, CaMsn4 and Mnl1 do not appear to play significant roles in responses to cellular stresses in *C. albicans*. (v) Expression of a protein fusion containing the Msn4<sub>DBD</sub> domain linked to the VP16 transcriptional activation domain (Fig. 9) did not lead to the activation of any stress-related functions in *C. albicans* (not shown). Indeed, no significant CaMsn4 targets were observed using this approach. Also, a SaLexA-CaMsn4 fusion showed no transcriptional activation in *C. albicans*. We are unable to exclude the possibility that the SaLexA-CaMsn4 fusion was aberrantly expressed, folded, or localized. However, the positive control, SaLexA-Gcn4, did activate transcription in this experimental system (Fig. 8). Hence, if CaMsn4 does retain functionality as a transcription factor in *C. albicans*, this functionality presumably depends on other factors not examined in this study.

Two potential targets of CaMsn4 were identified by transcript profiling and confirmed by Northern blotting (Fig. 5). The induction of *CaYPL088* in response to stress appeared to be dependent upon CaMsn4 (and, to a lesser extent, upon CaMnl1). In contrast, *CaYIR035* mRNA levels were constitutively elevated in cells lacking CaMsn4. CaMsn4 might act indirectly upon these genes. Nevertheless, these represent the first identified gene targets for CaMsn4.

On the basis of the above observations, we conclude that CaMsn4 and Mnl1 do not play significant roles in the stress responses examined. This conclusion is consistent with the view that there has been significant evolutionary divergence between *S. cerevisiae* and *C. albicans* with respect to their stress responses. Such divergence has probably been driven by the evolution of niche-specific environmental responses, because the environmental challenges posed to a fungal pathogen of

humans are likely to be quite distinct from those posed to a saprophytic fungus.

It is not surprising, therefore, that recent transcript-profiling experiments have revealed significant differences in the molecular responses of different fungi to stress. *Schizosaccharomyces pombe* and *S. cerevisiae* exhibit core transcriptional responses to a variety of different stresses, including heat, acid and alkali shifts, and osmotic and oxidative stresses (8, 9, 21). This core transcriptional response is reflected at the cellular level by the phenomenon of “cross-protection,” in which exposure to a mild dose of one form of stress protects the fungus against more severe doses of a quite different type of stress. Interestingly, these core transcriptional responses are regulated in different ways. *S. pombe* appears to exploit a common SAPK signaling pathway in which Sty1 activates its common set of stress genes (9), whereas *S. cerevisiae* uses different signaling pathways to activate its common set of stress genes (8, 9, 21). *C. albicans* differs from these benign fungi in that it did not display a common core transcriptional response to sublethal heat, osmotic, and oxidative stresses that induce such responses in *S. pombe* and *S. cerevisiae* (15). Instead, specific molecular responses to each stress were observed, and this was consistent with the lack of cross-protection provided by mild heat, osmotic, or oxidative stress (15). Hence, these three fungi clearly display specialized stress responses that presumably reflect their contrasting niches.

The situation is complicated by the potential involvement of dose-dependent stress-signaling networks in each fungus. For example, in *S. pombe*, the Sty1 pathway is activated by H<sub>2</sub>O<sub>2</sub> in a dose-dependent fashion via two distinct sensing mechanisms (49). Hence, it could be argued that CaMsn4 (and possibly Mnl1) might be required for responses to only certain doses of a particular stress. However, we observed no phenotypic effects of the *msn4/msn4* and *mnl1/mnl1* mutations following exposure to a wide range of salt, nutrient, or oxidative stresses. Therefore, stress signaling in *C. albicans* appears to have diverged to the extent that Msn2- and Msn4-like proteins no longer play significant roles.

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