The *Saccharomyces cerevisiae* RanGTP-Binding Protein Msn5p Is Involved in Different Signal Transduction Pathways

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ABSTRACT

In eukaryotes, control of transcription by extracellular signals involves the translocation to the nucleus of at least one component of the signal transduction pathway. Transport through the nuclear envelope requires the activity of an import or export receptor that interacts with the small GTPase Ran. We have cloned the *MSN5* gene of the yeast *Saccharomyces cerevisiae* that is postulated to encode one of these receptors. Msn5p belongs to a family of proteins with a conserved N-terminal sequence that acts as a RanGTP-binding domain. The results presented here provide genetic data supporting Msn5p involvement in several different signal transduction pathways. All of these pathways include changes in gene expression, and regulated nucleocytoplasmic redistribution of a component in response to external conditions has already been described in some of them. We have cloned *MSN5* following two different strategies. Msn5p was constitutively localized in the nucleus. Phenotypic analysis of the *msn5* mutant demonstrated that this protein participates in processes such as catabolite repression, calcium signaling, mating, and cell proliferation, as well as being involved in previously characterized phosphate utilization. Therefore, Msn5p could be a receptor for several proteins involved in different signaling pathways.

THE proper function of cellular processes requires continuous transport of molecules between the nucleus and cytoplasm. Numerous proteins are transported from the cytoplasm, where they are synthesized, to the nucleus. In addition, different types of RNA that are synthesized in the nucleus have to be transported to the cytoplasm before translation. The transport of molecules through the nuclear pore complexes has to be specific, so only the proteins that participate in nuclear function enter into the nucleus. Moreover, the possibility to modulate the nuclear localization of proteins can be used by the cell as a regulatory mechanism. During the last few years, nuclear translocation in response to ligands or environmental stimuli has been revealed as a common mechanism to regulate gene expression. In many signal transduction pathways regulated translocation of one or more specific proteins has been reported. For example, in mammalian cells the activation of mitogen-activated protein kinase cascades requires, besides the catalytic activation, a rapid relocalization of the kinases from the cytoplasm to the nucleus (Chen *et al.* 1992; Gonzalez *et al.* 1993). In the fission yeast *Schizosaccharomyces pombe*, stress conditions induce the nuclear localization of specific elements of the stress-activated protein kinase pathways (Gaits *et al.* 1998; Toone *et al.* 1998).

In *Saccharomyces cerevisiae* different pathways promote nucleocytoplasmic redistribution of transcription factors in response to external conditions. In yeast cells under oxidative stress, Yap1p is translocated into the nucleus (Kuge *et al.* 1997), and a variety of stress conditions such as heat shock, osmotic stress, and carbon-source starvation induce the nuclear accumulation of the zinc finger proteins Msn2p and Msn4p (Görner *et al.* 1998). Pho4p, a transcription factor necessary to induce a group of genes in response to phosphate starvation (Kaffman *et al.* 1994), shows a nuclear localization in phosphate-limiting conditions but when yeast are grown in phosphate-rich media, Pho4p is phosphorylated by the Pho80p-Pho85p kinase, resulting in a cytoplasmic localization (O'Neill *et al.* 1996).

In the presence of glucose many genes are repressed in yeast. Glucose starvation activates the serine/threonine kinase Snf1p that is required for the expression of glucose-repressed genes. Snf1p kinase interacts with its activating protein Snf4p and inhibits the repressor factor Mig1p. Mig1p is mainly localized to the nucleus in cells grown in high glucose concentration but is rapidly exported to the cytoplasm when glucose is removed (DeVit *et al.* 1997). The function of Mig1p may be regulated by phosphorylation since the level of phosphorylation of Mig1p is higher in the absence than in the presence of glucose (Treitel and Carlson 1995). The phosphorylation of Mig1p is regulated by the Snf1p

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protein kinase (Treitel *et al.* 1998) and could be reversed by the protein phosphatase Glc7p, which is required for glucose repression (Tu and Carlson 1995). According to this, a role for phosphorylation in establishing the localization of Mig1p has been suggested (DeVit *et al.* 1997).

Calcium signals regulate gene expression in many different cell types (Clapham 1995). In *S. cerevisiae*, Ca^{2+} and calmodulin activate the highly conserved serine/threonine phosphatase calcineurin, which is responsible for the induction of several genes implicated in different cellular functions. Activated calcineurin induces the expression of *PMC1* and *PMR1* (Cunningham and Fink 1996), which encode Ca^{2+}-pumping ATPases in the vacuole and Golgi, respectively (Rudolph *et al.* 1989; Antebi and Fink 1992; Cunningham and Fink 1994; Sorin *et al.* 1997). Ca^{2+}/calmodulin-bound calcineurin negatively regulates the vacuolar H^+/Ca^{2+} exchanger Vcx1p at the transcriptional and, mainly, post-transcriptional levels. Other genes regulated by calcineurin in response to high calcium concentrations are the Na^+-efflux pump *PMR2A* and *FKS2*, a β-1,3-glucane synthase required for cell wall integrity. The zinc finger protein Tcn1p/Crz1p has been identified as a calcineurin-dependent transcription factor that mediates changes in gene expression (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). Although the molecular mechanism is not yet known, one simple model would be that Tcn1p/Crz1p moves to the nucleus after dephosphorylation by calcineurin, since the nuclear localization of a Tcn1p/Crz1p-GFP fusion protein was found to be calcineurin dependent (Gerontides *et al.* 1999).

The nuclear accumulation of a protein depends on the balance of both import and export processes. The import often requires the presence of a nuclear localization signal (NLS), although it is possible the entry to the nucleus is through interaction with an NLS-containing partner. The export of some proteins depends on a short leucine-rich sequence called the nuclear export signal (NES), first identified in the HIV-1 Rev protein (Fisher *et al.* 1995). The small GTPase Ran plays a key role in the nucleocytoplasmic transport. Both import and export receptors involved in the transport of proteins with NLS or leucine-rich NES, respectively, interact with RanGTP and it is likely that the same feature occurs in the receptors of other nucleocytoplasmic transport pathways. In relation to this, Görlich *et al.* (1997) have identified a family of proteins whose members share an NH_2-terminal sequence that probably acts as an RanGTP-binding domain. The list of these factors includes some yeast proteins whose functions appear to be very different. In this article we describe the cloning and phenotypic characterization of *MSN5*, a gene that encodes a member of the RanGTP-binding protein family. The *MSN5* gene has been identified in two very different genetic screens: as a multicopy suppressor of the invertase defect in a Snf1p protein kinase mutant (Estruch and Carlson 1990) and as a gene required for the calcineurin-dependent induction of *PMC1* (Matheos *et al.* 1997). Moreover, recently, Msn5p has been shown as the nuclear export receptor of the transcription factor Pho4p (Kaffman *et al.* 1998). We show that Msn5p is a nuclear protein needed for the correct function of several cellular processes including carbon-source utilization, calcium tolerance, mating, and cyclin-specific functions. Our results suggest that Msn5p could be the nucleocytoplasmic transport factor of several cargos working in different signaling pathways.

MATERIALS AND METHODS

Yeast strains and genetic methods: The *S. cerevisiae* strains used in this study are listed in Table 1. Rich medium (YP) contains 1% yeast extract, 2% Bacto-peptone, and a 2% concentration of the indicated carbon source. Synthetic medium (S) consists of yeast nitrogen base (6.7 g/liter) lacking the appropriate amino acid and 2% of the indicated carbon source. To test the ability to suppress the *snf1-ts* growth defect, strains carrying different plasmids were spotted onto S-raffinose plates and incubated anaerobically in GasPaks (BBL, Microbiology Systems, Cockeysville, MD), or were grown overnight in YPD plates and then replica-plated on YP-sucrose containing 1 μg/ml of the respiratory inhibitor antimycin A. Carbon-source fermentation was scored on YPD or YP-galactose plates containing 2 μg/ml antimycin A. Growth kinetics in medium with raffinose as carbon source were done with cells grown in liquid YPD medium to early exponential phase and then transferred to S-raffinose. For mating tests, strains of different mating type were mixed together on YPD plates and grown overnight. Patches were replica-plated on synthetic medium selective for diploids and incubated at 30° for 2 days.

Isolation of the *MSN5* gene: The plasmid pEL335, carrying a yeast genomic DNA insert that includes the *MSN5* gene, was isolated by its ability to suppress the growth defect on raffinose shown by a *snf1-11ts* mutant (Estruch and Carlson 1990). A *snf1-11ts* mutant strain was transformed with a yeast genomic library in the multicopy plasmid YEp24 (Carlson and Botstein 1982). Transformants were replica-plated to S-raffinose medium and incubated anaerobically at 33°. Plasmid DNA was recovered from the raffinose fermenting transformants by passage through bacteria (Hoffman and Winston 1987). The isolation of *msn5* mutants by their inability to induce the expression of a *PMC1-lacZ* gene fusion in response to high calcium has been described in Matheos *et al.* (1997). Eleven of the recessive mutants isolated also showed mating defects and all of them were complemented by low copy plasmids carrying the *MSN5* gene.

Plasmids and gene disruptions: Plasmids pEL335D, -J, -P, and -Q contain the indicated genomic yeast DNA sequences cloned in YEp24 (Figure 1). In plasmid p335-Δ2::HIS3 the *MSN5* coding region has been deleted from the *Nsi*I site, at the position +689, to the *Eco*RI site, at position +2314, and replaced by the *HIS3* gene. In p335-Δ3::HIS3 the region deleted goes from a *Sma*I site generated by PCR, at the position +237, to the *Bgl*II site, at position +2331, and it has also been replaced by the *HIS3* gene. A high-dosage plasmid containing the *TCN1* gene (pDM9) was constructed by subcloning the *TCN1* locus from pDM3 (Matheos *et al.* 1997) into the multicopy plasmid B2205 (Cunningham and Fink 1994). Restriction fragments from p335-2::URA3, p335-Δ2::HIS3, and p335-Δ3::HIS3 were used to transform haploid and diploid

TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
MCY829	*MAT***a** *his3-Δ200 lys2-801 ura3-52*	M. Carlson
MCY1853	*MAT***a** *snf4-Δ2 his4-539 lys2-801 ura3-52*	M. Carlson
MCY2119	*MAT***a** *snf1-Δ10 ade2-101 his3-Δ200 trp1-Δ1 ura3-52*	M. Carlson
MCY2120	*MAT***a** *snf1-Δ10::snf1-11ts::TRP1 ade2-1his3-Δ200 trp1-1 ura3-52*	M. Carlson
MCY829msn5-2	*MAT***a** *msn5-2::URA3 his3-Δ200 lys2-801 ura3-52*	This work
MCY829msn5-Δ2	*MAT***a** *msn5-Δ2::HIS3 his3-Δ200 lys2-801 ura3-52*	This work
MCY829msn5-Δ3	*MAT***a** *msn5-Δ3::HIS3 his3-Δ200 lys2-801 ura3-52*	This work
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
K397	*MAT***a** *bar1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Nasmyth
K430	*MAT***a** *swi6::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Cunningham
K431	*MAT***a** *swi4::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Cunningham
K586	*MATα cln1::TRP1 cln2::LEU2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Cunningham
K589	*MATα cln2::LEU2 cln3Δ::URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Cunningham
K593	*MATα cln1::TRP1 cln3Δ::URA3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Cunningham
K1534	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1*	K. Nasmyth
PAY20	*MAT***a** *msn5-Δ3::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	This work
PAY21	*MATα msn5-Δ3::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	This work
PAY23	*MAT***a** *msn5-Δ3::HIS3 pmc1::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	This work
PAY23c	*MATα pmc1::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	This work
PAY61	*MAT***a** *MSN5::9MYC::TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	This work
PAY70	*MAT***a** *msn5-Δ3::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 bar1*	This work
DMY14	*MAT***a** *tcn1::KANMX3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	K. Cunningham
PAY100	*MATα swi4::LEU2 msn5-Δ3::HIS3 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1 ura3-1*[YCp *URA3 GAL::MSN5*]	This work

strains to yield, respectively, the *msn5* alleles *msn5-2::URA3*, *msn5-Δ2::HIS3*, and *msn5-Δ3::HIS3*. Disruptions of the chromosomal locus were confirmed by Southern blot analysis. Plasmid pBM95, containing the open reading frame of *MSN5* (*Sal*I-*Xho*I fragment) fused to the inducible *GAL10* promoter and cloned into pRS316, was generously given by M. Peter (ISREC, Switzerland).

Nucleic acids analysis: Restriction fragments were subcloned into pUC18 or pUC19 and sequenced by the method of Sanger *et al.* (1977) using a Sequenase 3.0 kit (U.S. Biochemical Corporation, Cleveland, OH) with the 17-nucleotide universal primer. The sequence was completed using serial deletion fragments and synthetic oligonucleotides. Standard methods were used for DNA and RNA analysis (Sambrook *et al.* 1989).

***In situ* immunolocalization:** To immunolocalize Msn5p, a tagged version was constructed by introducing nine copies of the Myc epitope immediately before the stop codon of *MSN5*. A PCR product containing two flanking sequences corresponding to the 3′ region of *MSN5*, nine copies of the Myc sequence, and the *Kluyveromyces lactis TRP1* marker was generated by using the primers PAmsn5a (5′-GATCCGAACATTG AAGACGGTGCTGTGGGTAATCTCTTTGATGACAACTCC GGTTCTGCTGCTAG-3′) and PAmsn5b (5′-TTTTCTCTAA TTTCATCTACACATCTACGTATATATGGCACCTTATTAC CTCGAGGCCAGAAGAC-3′) and the plasmid GA2265 (containing nine copies of *MYC* and the *K. lactis TRP1* gene in pUC19; W. Zaccharias and K. Nasmyth, unpublished results) as template. The PCR product was used to transform the W303-1A strain and the Myc tag insertion was confirmed by PCR. The production of the expected myc-fusion protein was checked by Western blot. For cellular localization of Msn5p-myc, overnight cultures were diluted to an OD_{600} of 0.3 in YPD and were grown to an OD_{600} of 1. Cells were then collected and treated for *in situ* immunofluorescence according to Nasmyth *et al.* (1990). To detect immunostaining of Msn5p-myc, mouse monoclonal antibody 9E10 (Gartner *et al.* 1998) was used at a 1:10 dilution, and the signal was detected by indirect immunofluorescence with FluoroLink Cy3-labeled goat anti-mouse (Amersham Life Science) at 1:200 dilution. Five minutes prior to microscopy, 4,6-diamidino-2-phenylindole (DAPI; final concentration 0.8 mg/ml) was added as DNA dye. Fixed cells were viewed with a Zeiss Axoplan 2 fluorescence microscope and images were scanned with a Quantix CCD camera using IP LAB software.

Tolerance experiments: The quantitative $CaCl_2$ tolerance assays were performed as described in Cunningham and Fink (1996). The concentration of $CaCl_2$ resulting in a 50% decrease in cell growth relative to unsupplemented cultures (IC50) was interpolated from linear plots of the ion tolerance data. For testing the tolerance of the *msn5* mutant to high calcium concentrations, cells grown on a YPD plate for 24 hr were streaked on YPD medium buffered at pH 5.5 with 0.005 m succinic acid (YPD buffered) and supplemented with $CaCl_2$ at the indicated concentrations. To test complementation of the *msn5* and *tcn1* growth defect in calcium, serial fourfold dilutions of exponential cultures in S-dextrose lacking uracil were spotted onto YPD-buffered medium supplemented with 200 or 350 mm $CaCl_2$. The W303-1A strain transformed with YEp24 and W303msn5-Δ3 and DMY14 strains transformed with plasmids YEp24, pDM9, and pE335Q were used. To test NaCl, LiCl, and pH tolerance, serial fivefold dilutions of exponential cultures in YPD were spotted in YPD solid medium containing the corresponding salt at the indicated concentra-

tion or buffered with 50 mm *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS) to yield pH 8.5.

Construction of the *swi4*, *msn5*, *GAL::MSN5* conditional lethal strain: Strain PAY21 (*msn5-Δ3::HIS3*) was transformed with plasmid pBM95 to uracil prototrophy and crossed with strain K431 (*swi4::LEU2*). The selected diploids were sporulated and asci were dissected in YP-galactose plates. Segregants prototrophic for leucine, histidine, and uracil were assumed to be of genotype *swi4::LEU2*, *msn5-Δ3::HIS3*, *pGAL::MSN5*. Three segregants from three different tetrads were isolated and used to analyze the vegetative growth in glucose and galactose media.

Enzyme assays: For assays of invertase, preparation of glucose-repressed and derepressed cultures and assays of secreted invertase activity were done as described in Estruch and Carlson (1990). The expression of *PMC1*, *PMR1*, *VCX1*, *PMR2A*, and *FKS2* was followed by assaying β-galactosidase activity in yeast transformed with pKC190, pKC199, pKC200, pKC201, and pDM5, respectively (Cunningham and Fink 1996; Matheos *et al.* 1997). Yeast cells were grown overnight in synthetic selective medium to an OD_{600} of 0.2–0.6 (early log phase) and then transferred to YPD-buffered medium and YPD-buffered medium supplemented with 200 mm $CaCl_2$. After 1 hr of incubation in these conditions, β-galactosidase activity was measured as described in Ausubel *et al.* (1994). Units of activity normalized for the OD_{600} of the culture were calculated as described in Miller (1972). Average standard deviations of β-galactosidase activity were determined from at least three independent experiments.

Nucleotide sequence accession number: The EMBL Nucleotide Sequence Database number for the *MSN5* sequence is X93302.

RESULTS

Isolation of the *MSN5* gene as multicopy suppressor of *snf1-ts* mutations: Plasmid pEL335 was recovered from a yeast genomic library (Carlson and Botstein 1982) as a suppressor of the raffinose growth defect of the *snf1-11ts* mutation as described in Estruch and Carlson (1990). The plasmid restores anaerobic growth of *snf1-11ts* and *snf1-12ts* mutants on raffinose at 33° but not at 37°. The plasmid also restores growth on medium containing sucrose plus the respiratory inhibitor antimycin A (Figure 1). The improved growth on raffinose and sucrose reflects increased expression of invertase in *snf1-11ts* cells transformed with pEL335 (Table 2). Higher increases in invertase values were obtained when cells were transformed with plasmid pEY335Q containing a shorter segment of cloned yeast DNA. This effect could be due to the higher number of copies per cell of pEY335Q in relation to pEL335 (Figure 1; Table 2). However, pEL335 did not suppress the growth on raffinose or invertase defects of *snf1-Δ10* and *snf4-Δ2* null mutants (results not shown; Table 2), suggesting that the suppression requires a certain level of Snf1p kinase activity.

To identify the suppressor gene on pEL335 we subcloned different restriction fragments into the multicopy vector YEp24 and tested their ability to suppress the growth defect of a *snf1-11ts* mutant on sucrose in the presence of antimycin A (Figure 1). The suppressor gene was delimited in a 5.2-kb region between the *Xho*I and *Hpa*I sites. The sequence of this region revealed a single complete open reading frame. The gene was designated *MSN5*, for *m*ulticopy *s*uppressor of *snf*.

Isolation of *msn5* mutants by their inability to induce the expression of a *PMC1-lacZ* gene fusion by high Ca^{2+} concentrations: *msn5* mutants were also identified in a genetic screen for mutants defective in calcium signaling (Matheos *et al.* 1997). Mutants were isolated by their inability to induce *PMC1-lacZ* expression during growth in high-Ca^{2+} conditions. The recessive mutants recovered from this genetic screen were placed into three complementation groups. The members of two of these groups were mutant alleles of the *CNB1* and *TCN1*/*CRZ1* genes encoding the calcineurin regulatory subunit and a calcineurin-dependent transcription factor, respectively (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). The members of the third complementation group (11 isolates) were complemented by low copy plasmids containing *MSN5* gene (see materials and methods).

Analysis of the suppressor gene and subcellular localization of Msn5p: The *MSN5* gene was sequenced and is predicted to encode a protein of 142.037 D (see materials and methods). Msn5p contains an N-terminal sequence motif related to the Ran-binding site of importin-β and binds to RanGTP *in vitro* (Görlich *et al.* 1997). Outside of this region, no homologies to other proteins were detected, although Msn5p is similar to other importin-β family members in size, isoelectric point (5.1 for Msn5p) and high helical region content (Görlich *et al.* 1997).

The expression of the *MSN5* gene was analyzed by Northern blot using the coding sequence as a probe. A 4-kb RNA was detected, which was equally abundant in cells grown in medium containing glucose, galactose, or raffinose as carbon source. The transcription of the *MSN5* gene is not induced by incubation in high concentrations of Ca^{2+} or Na^{+}, or by high pH (results not shown).

To investigate the intracellular location of the Msn5p, we constructed an epitope-tagged version of the *MSN5* gene (*MSN5::MYC*) containing nine tandem repeats of the Myc epitope at the C terminus (see materials and methods). The strain PAY61 carrying a genomic copy of *MSN5::MYC* behaved as a wild-type strain for all the conditions tested, indicating that the tagged Msn5p-myc protein is functional. By Western blot analysis using a total yeast protein extract, the anti-myc antibody recognized a single protein with the expected size for the Msn5p-myc fusion (results not shown). The subcellular distribution of Msn5p-myc was analyzed in PAY61 cells growing exponentially. Figure 2 shows that the CY3 signal corresponding to Msn5p-myc co-localized with the nuclear DAPI signal, indicating that Msn5p-myc is concentrated in the nuclei. There was also a slight CY3 fluorescence signal in the rest of the cell that is not observed in the control *MSN5* strain lacking the epitope tag (Msn5p; Figure 2). This staining indicates that a

Figure 1.—Maps of the *MSN5* locus and the *MSN5* overexpression and disruption plasmids. The arrow indicates the *MSN5* coding region (from 5′ to 3′ direction). The right side of the figure shows the growth in YP-sucrose containing antimycin A (2 μg/ml) of a *snf1-ts1* mutant strain transformed with the indicated plasmids. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hpa*I; Ha, *Hae*III; N, *Nsi*I; S, *Sac*II; Sp, *Spe*I; X, *Xho*I; Xb, *Xba*I.

minor proportion of Msn5p-myc is distributed throughout the cell. We have also examined the Msn5p-myc localization under heat stress (39°) and following exposure to high calcium (200 mm $CaCl_2$) or to pheromone (5 μm α-factor). Under all these conditions the distribution of Msn5p-myc was similar to that found in exponentially growing cells (results not shown).

Effect of the *MSN5* gene dosage on the utilization of nonrepressive carbon sources: To ascertain the phenotype of a *msn5* null mutation, we constructed three distinct disruptions (Figure 1). The *msn5-1::URA3* disruption was introduced into a wild-type diploid strain and the resulting heterozygous diploid was sporulated and subjected to tetrad analysis. Tetrads yielded four viable spores, indicating that the *MSN5* gene is not essential (results not shown). The *msn5-Δ2::HIS3* and *msn5-*

TABLE 2

Effect of the increased *MSN5* gene dosage and *msn5* deletion on invertase activity

	Invertase activity[a]	
Relevant genotype	Repressed	Derepressed
Wild type (YEp24)	<1	350
snf1-11ts (YEp24)	<1	19
snf1-11ts (pEL335)	<1	51
snf1-11ts (pEL335Q)	5	79
snf4-Δ2 (YEp24)	<1	2
snf4-Δ2 (pEL335)	<1	5
snf4-Δ2 (pEL335Q)	<1	11
snf1-Δ10 (pEL335)	<1	1
Wild type	<1	284
msn5-Δ3::HIS3	<1	171

[a] Strains carrying plasmids were grown in supplemented synthetic medium lacking uracil, and values are averages of assays of at least three transformants. Strains without plasmids were grown in rich medium. Both media contained 2% glucose. Derepression was done in the same media but containing 0.05% glucose for 3 hr at 30°. Invertase activity is expressed as micrograms of glucose released per 100 mg (dry weight) of cells.

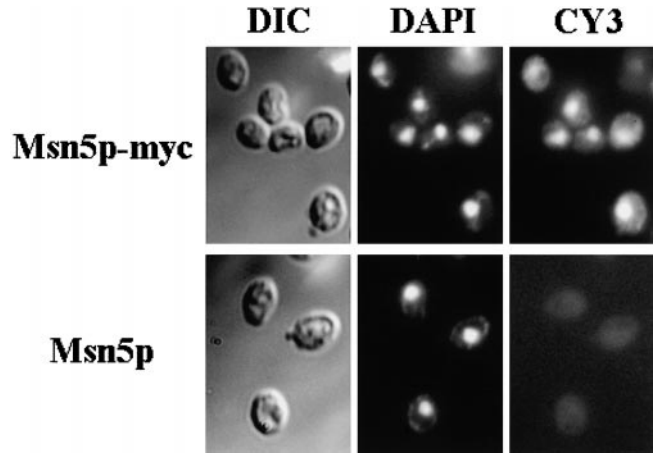

Figure 2.—A Msn5-myc fusion protein shows a predominant nuclear localization. W303-1A cells containing a myc-tagged version of *MSN5* gene in the genomic locus (PAY61 strain) were grown in YPD to mid-log phase and then treated for *in situ* immunofluorescence analysis (Msn5p-myc). Control wild-type cells with untagged Msn5p are included at the bottom (Msn5p). Photographs obtained by fluorescence microscopy show DAPI signal corresponding to nuclear and mitochondrial DNA (DAPI) and CY3 signal corresponding to Msn5p-myc localization (CY3). Overlaid photographs were obtained by light microscopy (DIC, differential interference contrast).

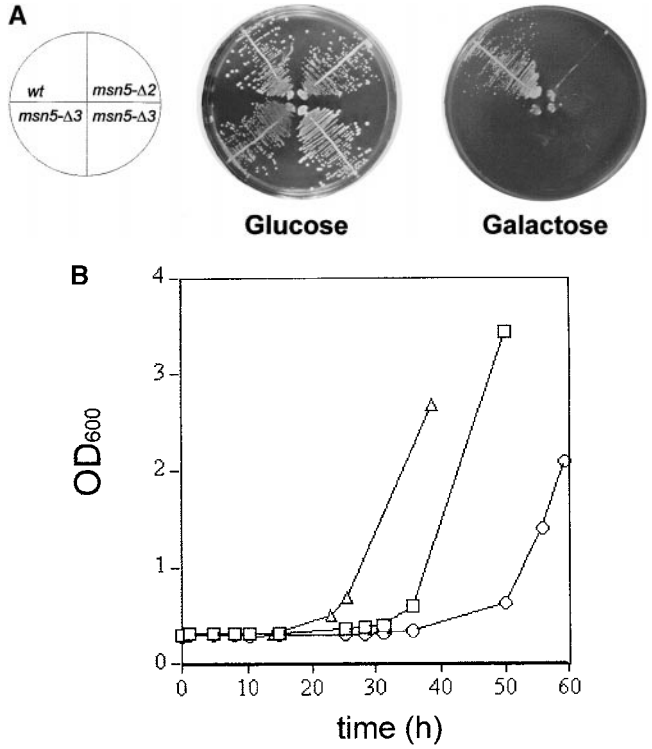

Figure 3.—*MSN5* functions in the utilization of nonrepressive carbon sources. (A) *msn5* growth defect in galactose. Growth of MCY829 (wt), MCY829msn5-Δ2 (*msn5-Δ2*), and MCY829msn5-Δ3 (*msn5-Δ3*) strains in YPD (glucose) and YP-galactose (galactose), in the presence of antimycin A (2 μg/ml). (B) Effect of the *MSN5* gene dosage in the lag phase observed after transferring cells from a glucose-containing medium to a medium with raffinose as sole carbon source. W303msn5-Δ3 transformed with YEp24 (circles) and W303-1A transformed with pEL335 (triangles) or YEp24 (squares) were grown in YPD to midexponential phase and then transferred to S-raffinose-uracil medium. Growth was measured as increase in the OD_{600}.

Δ3::HIS3 mutations were introduced into haploid strains to replace the wild-type allele.

We reasoned that since *MSN5* was a multicopy suppressor of defects in *snf1* mutants, its product could be involved in carbon-source utilization. In relation to this, *msn5* mutants showed a slow growth on galactose in the presence of antimycin A, but grew as well as the wild type on glucose, raffinose, and glycerol (Figure 3A; results not shown). Further analysis revealed that the galactose growth defect was background dependent. When the *msn5-Δ3::HIS3* mutation was introduced into W303-1A, which grows much more vigorously on galactose than do the previously used strains of the S288C genetic background, no defects in galactose utilization were observed (results not shown). When the *msn5* mutant was assayed for invertase, a twofold reduction relative to wild type was observed (Table 2). This reduction was not sufficient to cause a growth defect on raffinose.

We had previously noticed that when exponentially growing wild-type yeast cells are transferred from YPD to synthetic minimal medium containing raffinose as carbon source (S-raffinose), a long lag phase is observed, during which there is no significant increase of OD_{600} of the culture (Figure 3B; Martinez-Pastor and Estruch 1996). When this experiment was repeated using a *msn5* mutant strain, the lag phase observed was longer than that obtained for the wild-type strain (Figure 3B). In contrast, when we used wild-type cells transformed with a multicopy plasmid containing the *MSN5* gene (pEL335), a reduction in the time required to start exponential growth was observed (Figure 3B). Thus, the gene dosage of *MSN5* influences the time required to adapt the carbon-source metabolism from a repressed to a derepressed state. The transition is accelerated by a high *MSN5* dosage, whereas in the absence of *MSN5* a longer time is required.

Role of Msn5p in tolerance to high calcium concentrations: *msn5* mutants were identified in a selection for mutations that impaired the expression of a *PMC1-lacZ* fusion by high Ca^{2+} concentrations. The *PMC1* gene encodes a vacuolar Ca^{2+}-ATPase that is induced by calcineurin and is required for growth in high-Ca^{2+} conditions (Cunningham and Fink 1994). Therefore, we decided to analyze the tolerance to high concentrations of $CaCl_2$. As can be observed in Figure 4A, growth of the *msn5* mutant was defective at 100 mm and completely abolished at 200 mm. To establish the epistatic relations between *MSN5* and calcineurin we assayed the calcium tolerance (determined as the $CaCl_2$ concentration that causes a growth reduction of 50%, IC50 index) of several strains containing different combinations of mutations in the *MSN5*, *CNB1* (encoding the essential regulatory subunit of calcineurin) and the genes *PMC1* and *VCX1* that code for vacuolar calcium transporters regulated by calcineurin (Figure 4B). The calcium tolerance of the *msn5* mutant was similar to that found in the *pmc1* mutant and the combination of the two mutations did not produce any additive effect.

Calcineurin strongly inhibits the function of the H^+/Ca^{2+} exchanger Vcx1p at the post-transcriptional level (Cunningham and Fink 1996). The abolishment of this inhibition by deletion of the *CNB1* gene restored the Ca^{2+} tolerance in a *pmc1* mutant. We found that mutations in the *MSN5* and/or *PMC1* genes reduced calcium tolerance only in the absence of Vcx1p function (Figure 4B). The fact that Vcx1p inhibition by calcineurin occurs in the *msn5* mutants suggests that Msn5p works downstream of calcineurin in the calcium signaling pathway.

We next compared the calcium-induced expression of a *PMC1-lacZ* gene in a wild type and in a *msn5* mutant strain (Figure 5A). As expected by the strategy through which the *msn5* mutants were isolated (described above), the calcium-dependent induction of the reporter gene for *PMC1* was severely reduced in the *msn5* mutant strain. There are other genes that are activated by Ca^{2+} in a calcineurin-dependent way. The list in-

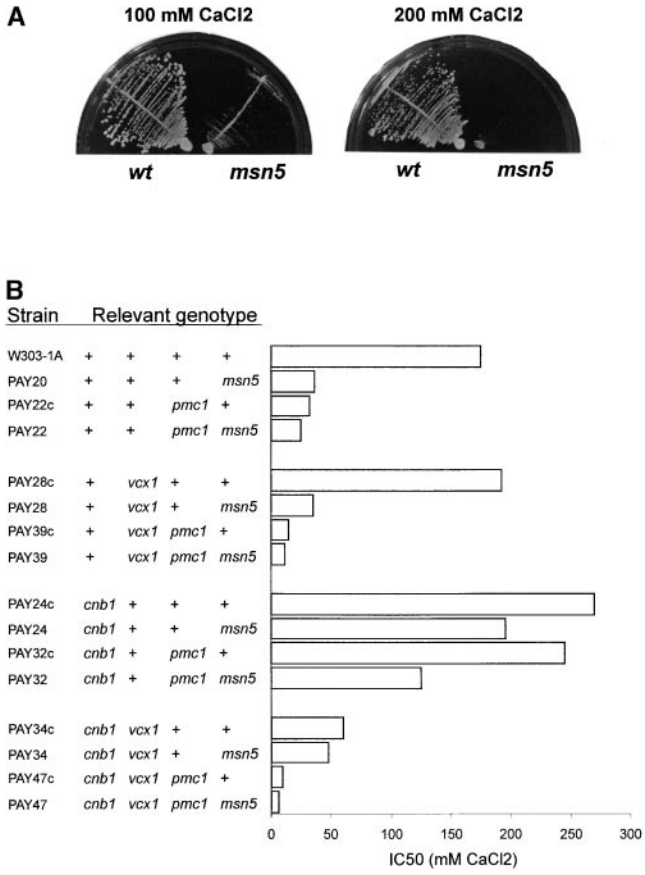

Figure 4.—*MSN5* functions in Ca^{2+} tolerance. (A) *msn5* growth defect in high-calcium media. Growth of the W303msn5-Δ3 (*msn5*) and W303-1A (*wt*) strains in YPD-buffered plates supplemented with $CaCl_2$ at the indicated concentrations. (B) Ca^{2+} tolerance assays of various yeast mutants involved in calcium signaling. All the strains used were isogenic to W303-1A and contained wild-type genomic copy (+) or mutated versions of the indicated genes (*cnb1*, *vcx1*, *pmc1*, *msn5*). The tolerance to Ca^{2+} is shown by the IC50 index, determined as described in materials and methods.

cludes the *PMR2A*/*ENA1* gene that encodes a P-type ion pump involved in Na^+ and Li^+ efflux (Rudolph *et al.* 1989; Haro *et al.* 1991); *FKS2*, which is involved in cell wall biosynthesis (Mazur *et al.* 1995); and *PMR1* encoding a Golgi Ca^{2+}-ATPase. To ascertain whether the effect of the *msn5* mutation on calcium-induced transcription was general, we investigated the requirement of Msn5p for the induction of *PMR2A*, *FKS2*, and *PMR1*. Figure 5A shows that the induction of a *PMR2A-lacZ* gene fusion by high Ca^{2+} was normal in a *msn5* mutant, although the basal expression is higher in the mutant strain. In contrast, induction of *FKS2-lacZ* and *PMR1-lacZ* by high Ca^{2+} concentration was defective in the *msn5* mutant. Thus, from these experiments we conclude that Msn5p is important for the induction of some but not all the calcineurin-dependent genes.

Besides *MSN5*, the search for mutants that failed to express *PMC1-lacZ* fusion during growth in high-Ca^{2+} conditions led to the identification of *TCN1*, encoding a zinc finger protein that functions as a calcineurin-dependent transcription factor. Tcn1p acts downstream of calcineurin and it is likely the last element in the branch of the calcium signaling pathway leading to the expression of genes such as *PMC1*, *PMR1*, *PMR2A*, and *FKS2* (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). To test for genetic interactions between *MSN5* and *TCN1*, we checked whether a multicopy plasmid carrying the *TCN1* or the *MSN5* gene was able to suppress the growth defect in high-Ca^{2+} media of a *msn5* or a *tcn1* mutant strain, respectively. As shown in Figure 5B the calcium-sensitive phenotype of the *msn5* mutant was partially suppressed by overexpression of the *TCN1* gene. However, no suppression was observed when the *MSN5* gene was overexpressed in a *tcn1* mutant strain (Figure 5C). One could expect these results if Msn5p acted upstream or parallel to Tcn1p in the calcineurin pathway.

***msn5* mutants are sensitive to high temperatures, high Na^+, Li^+, and Mn^{2+} concentrations, and alkaline pH and show mating defects:** In addition to the defects in carbon-source utilization and calcium sensitivity, *msn5* mutants exhibit many phenotypes. The strain lacking the *MSN5* gene is more sensitive than the isogenic wild-type strain to several stresses, including short exposures to high temperatures (Figure 6A), high concentrations of Na^+ or Li^+, or high pH (Figure 6B). The salt defect shown by the *msn5* mutant was specific for Li^+ and Na^+ ions and was not a consequence of the high osmolarity of the medium, because supplements of either 1.2 m KCl or 1.8 m sorbitol did not inhibit growth (results not shown). Other phenotypes associated with the *msn5* mutation include slow growth at high (37°) and low (14°) temperatures and sensitivity to high Mn^{2+} concentrations (results not shown). However the sensitivity to oxidative stress of a *msn5* mutant was similar to the wild type (results not shown).

Furthermore, *msn5* mutants have mating defects. Figure 6C shows that diploid formation between a *msn5* mutant and a wild-type strain is severely reduced, whereas the mating efficiency between two *msn5* mutant strains is almost zero. The partial mating defects shown by the *msn5* mutants could be related to a partial defect in pheromone response detected by halo assays (Figure 6D).

***MSN5* gene is essential in the absence of *CLN1* and *CLN2* cyclin genes and in the absence of the transcription factor SBF:** G_1 cyclins Cln1p and Cln2p are redundant and differ from Cln3p in their structure and regulation. At least one of the three G_1-specific cyclins is necessary for the activation of Cdc28 kinase and the execution of Start at the beginning of a new cell cycle (reviewed in Cross 1995). Recently, new functions for Cln1p and Cln2p in proliferation have been suggested; Cln1/Cdc28 and Cln2/Cdc28 seem to play direct roles in cytokinesis and bud emergence (Benton *et al.* 1997). We investigated the possible genetic interaction be-

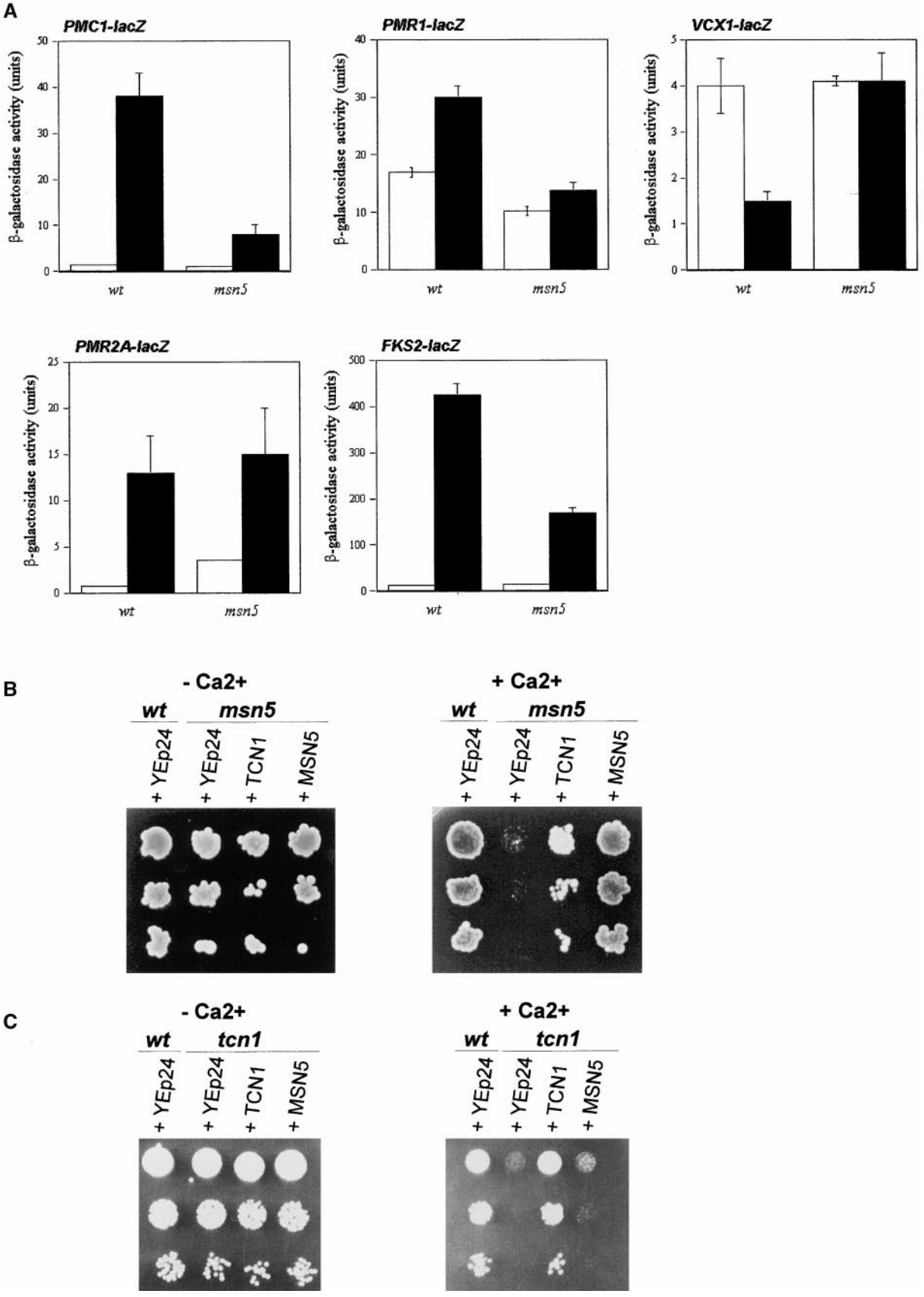

Figure 5.—(A) Calcium-induced expression of the calcineurin targets in the *msn5* mutant. W303-1A (wt) and W303msn5-Δ3 (*msn5*) cells carrying the indicated *lacZ* gene fusion (plasmids pKC190, pKC199, pKC200, pKC201, and pDM5, from top left to bottom right) were grown on S-dextrose-uracil medium to early log phase and then transferred to YPD-buffered (open bars) or YPD-buffered medium supplemented with 200 mm $CaCl_2$ (solid bars). β-Galactosidase activity was measured after 1 hr of

tween *MSN5* and *CLN* genes by combining *msn5* with *cln1*, *cln2*, and *cln3* mutations. By crossing a *msn5* mutant strain with a strain carrying mutation in the *CLN1* and *CLN2* genes, no segregants with the phenotype *msn5, cln1, cln2* were obtained from 20 tetrads analyzed (Table 3). This result suggests that the *msn5* mutation is synthetic lethal with mutations in the *CLN1* and *CLN2* genes. By crossing *msn5* with a double *cln2*, *cln3* mutant strain, it was possible to obtain spores with genotypes corresponding to the triple mutant, although the size of these colonies was substantially smaller than the wild type or double mutant strains (results not shown). Finally, no growth defects were observed in a *msn5*, *cln1*, *cln3* strain (results not shown). These results indicate that Msn5p shares an essential function with the cyclins Cln1p and Cln2p, perhaps being required for Cln3p function.

The activation of *CLN1* and *CLN2* expression is regulated by Cln3p-Cdc28p through the transcription factor SBF whose subunits are encoded by the *SWI4* and *SWI6* genes (reviewed in Koch and Nasmyth 1994). These two genes are essential in cells that lack *CLN1* and *CLN2*, having Cln3p as the only G_1 cyclin (Dirick and Nasmyth 1991). We crossed strains with single *swi4* and *swi6* mutations and a *msn5* mutant strain to determine synthetic interactions. We did not find any segregant with the markers corresponding to *msn5*, *swi4* or *msn5*, *swi6* double mutants (Table 3), suggesting that the lack of Msn5p in the absence of any of these SBF transcription factors causes lethality or prevents the germination of the ascospores. To distinguish between these possibilities a conditional *msn5* mutation was constructed by placing the coding region of the *MSN5* gene under the control of the regulatable *GAL* promoter. A yeast strain carrying the *GAL::MSN5* allele in combination with mutations in the *MSN5* and *SWI4* genes was constructed (see materials and methods). This strain depended on galactose for growth (Figure 7), indicating that Msn5p is needed to maintain vegetative growth when the transcription factor Swi4p is absent. In accordance with this result, a *msn5* mutant strain carrying a thermosensitive *swi4* allele was unable to grow at the restrictive temperature of 37° (J. C. Igual, personal communication).

DISCUSSION

The response to variations in environmental conditions or to extracellular signals often requires changes in gene expression. To achieve this, the signal system has to be linked to the nuclear transcription machinery; therefore, the translocation of one or more components of the signal transduction pathway to the nucleus is necessary. In the past few years many reports have revealed that the entry or exit of these components can be a regulatory step (reviewed in Wilkinson and Millar 1998; Nigg 1997). Independently, a superfamily of proteins that could act as transport factors has been identified (Görlich *et al.* 1997). These proteins are involved in very different cellular functions, suggesting that they would act in specific and independent nuclear transport pathways. In the yeast *S. cerevisiae*, 13 proteins have been proposed to belong to the family of nucleocytoplasmic transport factors. Here, we describe the cloning of one of these, Msn5p, using very different genetic strategies, suggesting that it could be involved in specific cellular processes. In agreement with this, *msn5* mutants show a variety of phenotypes, including carbon-source utilization defects and sensitivity to high concentrations of ions, severe heat shock, and high pH. Moreover, they are partially sterile and show synthetic lethality with a double mutant *cln1, cln2*. This collection of heterogeneous phenotypes makes it unlikely that Msn5p acts in a specific signal transduction pathway and would be more compatible with a role in a general regulatory mechanism common to the different pathways. The recent identification in Msn5p of a conserved N-terminal domain shared by proteins with RanGTP-binding activity suggests that Msn5p could participate in the nucleocytoplasmic transport. The localization of the Msn5p in the nucleus is consistent with a function in the transport of molecules through the nuclear envelope. We have not identified any obvious nuclear localization signal in the Msn5p sequence, although it could be maintained in the nucleus by interacting with nuclear components. Thus, it has been found that members of the same protein family are able to interact both with RanGTP and with nuclear pore complexes, although for Msn5p only the interaction with RanGTP has been proved (Görlich *et al.* 1997, and references cited therein). We think it is likely that the diversity of phenotypes associated with the *msn5* mutation are related to defects in the transport of proteins to or from the nucleus and that this role could also explain why *MSN5* was isolated using different genetic screens. Moreover, O'Shea's group has shown that Msn5p is the export receptor of the transcription factor Pho4p. Msn5p is required for nuclear export of Pho4p when yeast are grown in phos-

incubation in these conditions. (B) Suppression of the growth defect in high-calcium media of the *msn5* mutant by overexpression of the *TCN1/CRZ1* gene. (C) Overexpression of the *MSN5* gene cannot suppress the growth defect in high-calcium media of the *tcn1* mutant. In B and C several fourfold dilutions were prepared from exponentially growing cultures in S-dextrose-uracil medium and 10-μl aliquots were spotted onto YPD plates ($-Ca^{2+}$) and YPD-buffered plates ($+Ca^{2+}$) supplemented with 200 mm $CaCl_2$ (B) or 350 mm $CaCl_2$ (C). The strains used were W303-1A (*wt*), W303msn5-Δ3 (*msn5*), and DMY14 (*tcn1*) transformed with YEp24, pDM9 (*TCN1*), and pE335Q (*MSN5*) plasmids.

phate-rich medium and binds *in vitro* to phosphorylated Pho4p in the presence of RanGTP (Kaffman *et al.* 1998).

We identified *MSN5* as a multicopy suppressor of thermosensitive mutations in the *SNF1* gene. *SNF1* encodes a protein kinase required for derepression of the glucose-repressible genes. In our screening *MSN5* was isolated because its overexpression increases the invertase activity of a *snf1-ts* mutant to levels that allow the cells to grow in a medium containing raffinose as sole carbon source. In the yeast *S. cerevisiae*, invertase is encoded by *SUC2*, a well-characterized example of glucose-repressible gene. The transcriptional repressor factor Mig1p binds to the *SUC2* promoter and recruits the general repression complex Ssn6p-Tup1p (Keleher *et al.* 1992). DeVit *et al.* (1997) have found that the subcellular localization of Mig1p depends on glucose concentration; Mig1p is nuclear localized in the presence of glucose and is mainly cytoplasmic at low glucose concentrations. Recently, it has been found that Msn5p is required to export Mig1p from the nucleus (M. J. DeVit and M. Johnston, personal communication), suggesting an explanation about how *MSN5* can act as multicopy suppressor of *snf1-ts* mutations. Increased dosage of *MSN5* would favor the exit of Mig1p from the nucleus, relieving the transcriptional repression. Whether Msn5p directly interacts with Mig1p or the effect is indirect remains to be determined; but, in any case, the dependence of the export of Mig1p on Msn5p would require a certain level of Snf1p protein kinase activity since overexpression of *MSN5* does not suppress a *snf1* null allele. Since the phosphorylation of Mig1p is regulated by the Snf1p protein kinase (Treitel *et al.* 1998) it is possible that only the phosphorylated Mig1p would be a substrate of the Msn5p-dependent transport system. On the other hand, the fact that *SUC2* expression is still regulated in a *msn5* mutant strain indicates that other modes of regulation besides the subcellular localization are involved in Mig1p function. This situation corresponds to that of transcription factor Pho4p, wherein the interaction between Msn5p and Pho4p is regulated by the specific phosphorylation of two serine residues of Pho4p and, although in a *msn5* mutant strain Pho4p is constitutively localized to the nucleus, Pho4p-dependent transcription is not induced in phosphate-rich me-

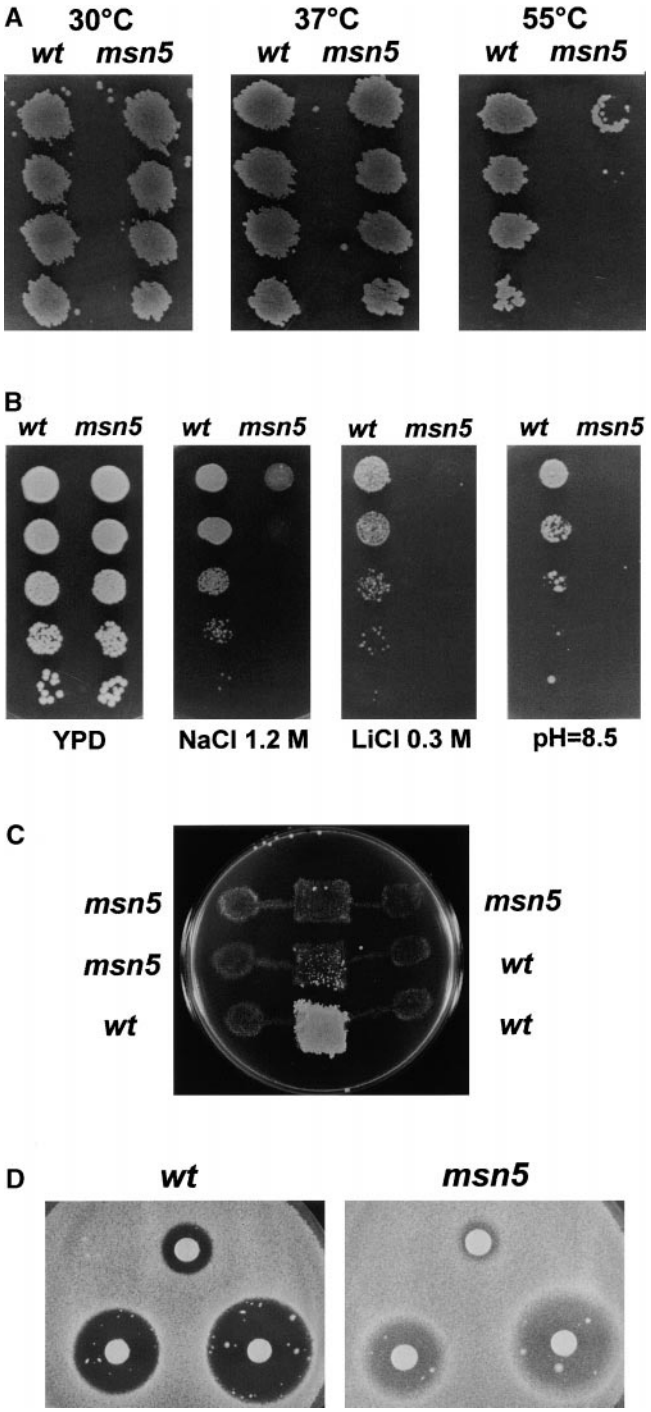

Figure 6.—Other phenotypes exhibited by the *msn5* mutant. (A) Sensitivity to heat-shock stress. Serial fivefold dilutions of W303-1A (*wt*) and W303msn5-Δ3 (*msn5*) strains were spotted onto YPD plates and were grown overnight at 30°. Three replicas were made on YPD plates that were incubated for 30 min at the indicated temperatures and then transferred at 30° where they were maintained for 2 days. (B) Sodium, lithium, and basic pH sensitivity of the *msn5* mutant strain. Serial fivefold dilutions of exponentially growing MCY829 (*wt*) and MCY829msn5-Δ3 (*msn5*) cells were spotted onto YPD plates, YPD plates supplemented with NaCl and LiCl at the indicated concentrations, and YPD plates buffered to pH 8.5 with TAPS. (C) Partial sterility of the *msn5* mutant strain. The ability to mate was tested by sexual crosses between the strains W303-1A transformed with YEp24 (*wt*) and PAY23c (*wt*) (bottom); W303msn5-Δ3 transformed with YEp24 (*msn5*) and PAY23c (*wt*) (middle); and W303msn5-Δ3 transformed with YEp24 (*msn5*) and PAY23 (*msn5*) (top). (D) Halo assays were performed as described in materials and methods by spotting α-factor (top, 0.05 μg; bottom left, 0.5 μg; bottom right, 2 μg) directly onto freshly applied lawns of K397 (*wt*) and PAY70 (*msn5*) strains.

TABLE 3

Synthetic interaction between *msn5* and G_1 cyclins and SBF transcription factors

Genetic cross[a]	No. tetrads analyzed	No. double/triple mutant spores		
		Spore genotype	No. expected[b]	No. obtained
msn5 × *cln1 cln2*	20	*msn5 cln1 cln2*	9	0
msn5 × *cln2 cln3*	11	*msn5 cln2 cln3*	10	8
msn5 × *cln1 cln3*	18	*msn5 cln1 cln3*	9	9
msn5 × *swi4*	22	*msn5 swi4*	25	0
msn5 × *swi6*	22	*msn5 swi6*	16	0

[a] The strains used in this experiment were as follows: PAY20 (*msn5*) for the mating with G_1-cyclin mutants, strain K586 (*cln1 cln2*), strain K589 (*cln2 cln3*), and strain K593 (*cln1 cln3*); PAY21 (*msn5*) for the mating with SBF transcription factors mutants, strain K431 (*swi4*), and strain K431 (*swi6*).

[b] This number refers to the spores from tetrads where the analysis of the markers of the surviving spores made it possible to determine exactly which spores had the indicated double or triple mutant genotype.

dium due to additional mechanisms that regulate Pho4p activity (Komeili and O'Shea 1999).

In a genetic screen described here and previously (Matheos *et al.* 1997), two new genes, *TCN1*/*CRZ1* and *MSN5*, were identified as putative members of the calcineurin pathway. Tcn1p is a transcriptional activator, acting downstream of calcineurin, required for regulation by calcium of *PMC1*, *PMR1*, and *FKS2* genes. One simple model to relate Tcn1p and Msn5p in the calcium signaling pathway could be that calcineurin dephosphorylates Tcn1p and/or other components of the pathway in such a way that the subcellular localization becomes modified, Msn5p being involved in the transport of the regulated component. For instance, similar to the situation found for Mig1p, Msn5p might be involved in the export of a specific transcription repressor or, on the contrary, it could be required for the nuclear translocation of Tcn1p. Each of these two possibilities could explain the sensitivity of the *msn5* mutant to high calcium, caused by the defective induction of *PMC1*. In a similar way, the Mn^{2+} hypersensitivity observed in the *msn5* mutants could be explained by a defect in the activation of the manganese tolerance factor *PMR1*, whose induction by Ca^{2+} also depends on Tcn1p

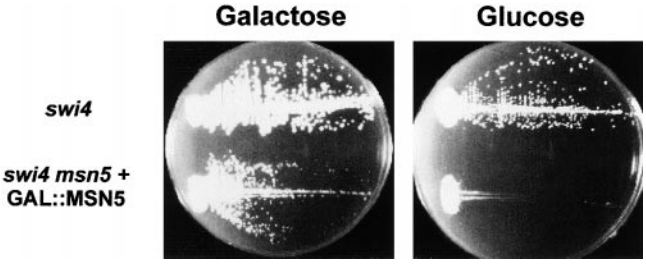

Figure 7.—Msn5p is required for vegetative growth in a *swi4* mutant strain. The strain PAY100 (*swi4 msn5 pGAL::MSN5*) carrying mutations in the *MSN5* and *SWI4* genes and a centromeric plasmid (pBM95) containing a *GAL::MSN5* construct was streaked on YPD (glucose) and YP-galactose (galactose) plates. Strain K431 (*swi4*) was used as control.

(Matheos *et al.* 1997). However, although the *msn5* mutants are also hypersensitive to high Na^+ concentration, we have not found any defect in the induction of the most important factor in the salt tolerance, *PMR2A*. This gene exhibits a complex regulation by at least three independent signaling pathways (Marquez and Serrano 1996). Our results point to a different calcium regulation between the Na^+-ATPase Pmr2Ap and the Ca^{2+}-ATPase-encoding genes *PMC1* and *PMR1*. This could be due to the existence of a repressor, whose export from the nucleus would be Msn5p dependent, acting on *PMC1* and *PMR1*, but not on *PMR2A*. This hypothesis is compatible with the partial complementation of the *msn5* calcium sensitivity by the overexpression of *TCN1*. Nevertheless, other models are possible and the *msn5* mutant sensitivity to Na^+ remains to be explained.

Another phenotype shown by the *msn5* mutants is an almost complete sterility. As the halo assay results suggest, the mating incapacity is likely a consequence of the defective induction of the pheromone response pathway. This is one of the best-characterized signal transduction pathways (for reviews, see references in Sprague and Thorner 1992), although little is known about the mechanisms by which the mating signal is transferred from the cytoplasm to the nucleus. The pheromone signaling starts with the pheromone receptor localized in the cell membrane and coupled to a G protein. The signal transduction pathway culminates in arrest of the cell cycle, changes in gene expression, and altered cell polarity and morphology. Some of the latest targets of the pathway responsible for these events are the transcriptional activator Ste12p (Song *et al.* 1991; Elion *et al.* 1993), the repressors Dig1p, Dig2p (Cook *et al.* 1996; Tedford *et al.* 1997), and the cyclin-dependent kinase inhibitor Far1p (Chang and Herskowitz 1990; Peter *et al.* 1993). Dig1p and Dig2p are localized in the nucleus, physically associated with Ste12p (Cook *et al.* 1996; Tedford *et al.* 1997). Far1p is localized in the nuclei in the absence of mating pheromone but partially

relocalizes from the nuclei to the cytoplasm in the presence of pheromone (Butty *et al.* 1998). In between the pheromone membrane-bound receptor and those nuclear targets, the signal propagates through a MAP kinase cascade composed of the MEKK Ste11p, the MEK Ste7p, and the MAPK Fus3p (Gartner *et al.* 1992; Errede *et al.* 1993; Neiman and Herskowitz 1994). The pathway requires Ste5p, a scaffold protein that binds each of the kinases of the cascade (Choi *et al.* 1994; Marcus *et al.* 1994; Printen and Sprague 1994). Pheromone stimulates translocation of Ste5p to the cell surface but Ste5p is found mainly in the nucleus before signaling (Pryciak and Huntress 1998). With this scenario, the sterility of the *msn5* mutants could be explained by a defect in the accumulation of the activator Ste12p in the nuclei or the export of the repressors Dig1p, Dig2p from the nuclei. However, the fact that the *dig1*, *dig2*, *msn5* triple mutant is able to activate *FUS1* transcription but still shows mating defects (P. M. Alepuz and G. Ammerer, unpublished results) argues against these possibilities. This result indicates that Ste12p is localized in the nucleus in the absence of Msn5p and that the cause of the *msn5* sterility is not a defect in the exclusion of Dig1p, Dig2p from the nucleus. On the contrary, a reasonable explanation for the mating defect of the *msn5* mutants is that Msn5p could be implicated in the transport of Far1p and/or Ste5p proteins from the nucleus to the cytoplasm in response to pheromone.

Finally, we have found a synthetic lethality between the *msn5* mutation and *cln1*, *cln2* mutant and *swi4* and *swi6* mutants. Some other genes have been described to show synthetic lethality in the absence of the G_1 cyclins Cln1p and Cln2p: *BUD2*, the negative regulator of the GTPase Bud1p, that is necessary for the correct choice of the bud site; *CLA4*, the Cdc42p-activated kinase working in cytokinesis; and *MPK1*, the MAP kinase of the Pkc1 pathway implicated in bud growth. Thus, a function in bud emergence and morphogenesis has been proposed for Cln1p and Cln2p and their transcriptional regulatory factors Swi4p/Swi6p (Benton *et al.* 1993, 1997; Cvrckova and Nasmyth 1993; Gray *et al.* 1997; Igual *et al.* 1997). Our results suggest that Msn5p could also be implicated in cell proliferation. The likely role of Msn5p as exportin suggests that this protein could be responsible for the export from the nucleus of an uncharacterized proliferation factor.

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