Increased dosage of the *MSN1* gene restores invertase expression in yeast mutants defective in the SNF1 protein kinase

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ABSTRACT

The SNF1 protein kinase is required for expression of the invertase gene in response to glucose deprivation in *Saccharomyces cerevisiae*. We selected for genes that in multicopy suppress the invertase defect of temperature-sensitive *snf1* mutants. Increased dosage of the *MSN1* gene restores high-level, regulated invertase expression in *snf1-ts* mutants, and disruption of *MSN1* in the wild type reduces invertase expression a fewfold. *MSN1* gene dosage does not affect SNF1 protein kinase activity in vitro. *MSN1* encodes a 43-kilodalton protein, and a *MSN1*- β -galactosidase fusion protein was localized in the nucleus. A LexA-*MSN1* fusion protein, when bound to a *lexA* operator, activates transcription of an adjacent promoter. In vitro synthesized *MSN1* protein exhibits weak, nonspecific DNA-binding activity.

INTRODUCTION

The *SNF1* gene of *Saccharomyces cerevisiae* encodes a protein-serine/threonine kinase that is required to release glucose-repressible genes from repression (1,2). The SNF1 protein kinase affects expression of many genes because glucose repression is a major regulatory mechanism in yeast. However, the precise function of the kinase in the regulatory pathway is not clear: the proteins that affect kinase activity, the targets of the kinase, and the proteins that directly mediate the effects on gene expression are largely unknown. Thus far, only one such protein has been identified. The *SNF4* gene encodes a protein that is physically associated with the SNF1 kinase and that is required for maximal kinase activity in vitro (2,3).

We report here the application of a genetic strategy to identify genes that encode products functionally related to the SNF1 protein kinase. We selected for genes that in multicopy suppress defects of temperature-sensitive *snf1* (*snf1-ts*) mutants. Temperature-sensitive mutations in *SNF1* cripple, but do not completely destroy, the SNF1 kinase activity, and thus allow selection of multicopy suppressors that are only partially effective.

One can imagine various mechanisms by which increased gene dosage could compensate for a defect in the kinase. For example, increased expression of an activator of the SNF1 kinase could elevate the activity of the mutant kinase, or overexpression of a target of the kinase could compensate for reduced kinase activity.

In this work, we selected for genes that in multicopy restore expression of the glucose-repressible *SUC2* gene, which is dependent on *SNF1* function. *SUC2* encodes invertase, a periplasmic enzyme that hydrolyzes sugars such as sucrose and raffinose. We could thus select for multicopy plasmids that restore the ability of *snf1-ts* mutants to grow on raffinose.

We report here the isolation and sequence of the *MSN1* (multicopy suppressor of *snf*) gene. Increased *MSN1* gene dosage restored high-level invertase expression in *snf1-ts* mutants, and disruption of *MSN1* reduced derepression of invertase in the wild type. We show that a *MSN1*- β -galactosidase fusion protein is localized in the nucleus, that a LexA-*MSN1* fusion protein activates transcription when bound to DNA, and that the *MSN1* protein has DNA-binding activity.

MATERIALS AND METHODS

Strains and genetic methods

Strains of *Saccharomyces cerevisiae* are listed in Table 1. The *snf1-ts* mutations were isolated on plasmids and then integrated in single copy at the *snf1- Δ 10* locus of MCY2119 (F.E. and M.C., unpublished). Standard methods were used for genetic analysis (4) and transformation (5). Growth on different carbon sources was scored by spotting cell suspensions on solid media and incubating anaerobically as described previously (6). Growth on glycerol was scored aerobically.

Isolation of multicopy suppressors of *snf1-ts* mutations

Strains carrying the *snf1-11ts* and *snf1-12ts* alleles were transformed with a yeast genomic library (7) in the multicopy vector YEp24 (8). A total of 30,000 Ura⁺ transformants were replica plated to synthetic medium lacking uracil and containing

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Table 1. List of *Saccharomyces cerevisiae* strains

Strain	Genotype
MCY730	<i>MATa ade2-101 ura3-52 lys2-801 his3-Δ200 trp1-Δ1 SUC2</i>
MCY829	<i>MATα ura3-52 lys2-801 his3-Δ200 SUC2</i>
MCY1093	<i>MATa his4-539 ura3-52 lys2-801 SUC2</i>
MCY1094	<i>MATα ura3-52 ade2-101 SUC2</i>
MCY1845	<i>MATα snf1-Δ10 ade2-101 ura3-52 SUC2</i>
MCY1853	<i>MATa snf4-Δ2 his4-539 ura3-52 lys2-801 SUC2</i>
MCY2119	<i>MATa snf1-Δ10 ade2-101 his3-Δ200 ura3-52 trp1-Δ1 SUC2</i>

raffinose as the carbon source, and plates were incubated anaerobically at 33°C. Plasmid DNA was recovered from raffinose-fermenting (Raf⁺) transformants by passage through bacteria. Plasmids carrying *SNF1* were identified by restriction analysis, and the remaining plasmids were retested by transforming the *snf1-ts* strains.

Nucleotide sequence analysis

Restriction fragments were subcloned into M13mp18 and M13mp19 (9) and sequenced by the method of Sanger et al. (10) with the 17-nucleotide sequencing primer (Amersham) and oligonucleotides complementary to previously determined sequences. The sequence of both strands was determined for the region -136 to +1401.

Construction of disruptions

Standard methods were used for manipulation and analysis of DNA (11). pEB312-Δ1::TRP1 and pEB312-Δ1::URA3 were constructed by first subcloning the *EcoRI* fragment of pEL312 in YEp24 at the *SmaI* site (pEY312C) and then transferring the *StuI-BamHI* fragment to pUC19 (12). The resulting plasmid, pUC312, contains the *EcoRI-BamHI* fragment of *MSN1*. The *HpaI-MscI* fragment of pUC312 was replaced with the *URA3* gene on a 1.1-kb *HindIII* fragment or the *TRP1* gene on the *ScaI-PvuII* fragment from pRS304 (13). The *SacI-BamHI* fragment from pEB312-Δ1::URA3 was used to replace (14) the wild-type sequence on one chromosome of the diploid MCY1093×MCY1094. The *TRP1* insertion was similarly introduced into MCY730, which was then crossed to obtain segregants. Constructions were confirmed by blot hybridization analysis.

Construction of *lacZ* and *lexA* fusions

A gene fusion between codon 298 of *MSN1* (at the *HpaI* site) and *lacZ* was constructed in the vector YEp358 (15), generating pMSN1(298)-*lacZ*. To construct pLexA-MSN1, the *MluI-EcoRI* fragment of *MSN1* was made blunt and cloned in the blunted *EcoRI* site of pSH2-1 (16).

Synthesis of MSN1 protein in vitro

The sequence from codon 39 to the end of the gene was first placed under control of the T7 promoter. Starting with pUC312, which contains the *EcoRI-BamHI* fragment of *MSN1* in pUC19, we inserted an 8-nucleotide *NcoI* linker into the *MluI* site at codon 39 so that the ATG of the *NcoI* site was in frame with the *MSN1* ORF. The *NcoI-EcoRI* fragment containing *MSN1* (the *EcoRI* site was derived from the pUC19 polylinker) was then cloned into the polylinker of the Bluescript KS vector (Stratagene), which had been modified by inserting a *NcoI* linker into the *SmaI* site.

The resulting plasmid is pEB312. *XhoI*-linearized pEB312 DNA (5 μg) was transcribed with T7 polymerase. RNA synthesis and capping were carried out as described previously (17). The RNA was translated with [³⁵S]methionine in a wheat germ lysate (Promega Biotec) according to the instructions of the supplier.

DNA binding assay

The procedure of Hope and Struhl (18) was used with minor modifications. Binding reactions were carried out in 15 μl of 50 mM Tris-HCl, pH 7.7, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride, and contained 2 μg of poly(dI.dC). Fragments of the *SUC2* 5' region cloned in pUC19 were released from the vector by digestion with *EcoRI* and *HindIII* and filled with Klenow fragment; this DNA was added to a final concentration of 9 nM. The in vitro translation reaction (1 μl) was then added, and the mixture was incubated for 20 min at room temperature. Samples were loaded on a 6% polyacrylamide gel in 45 mM Tris-borate buffer (pH 8.3). The gel was fixed, treated with en³Hance and autoradiographed.

Enzyme assays

Preparation of glucose-repressed and derepressed cultures and assays of secreted invertase activity were as described (6). β-Galactosidase activity was assayed in permeabilized cells (19), and units of activity normalized for the O.D.₆₀₀ of the culture were calculated according to Miller (20).

RESULTS

Selection for genes that in multicopy suppress *snf1-ts* mutations

Mutants carrying the *snf1-11ts* and *snf1-12ts* alleles show reduced growth relative to wild type at 33°C and no growth at 37°C on raffinose (F.E. and M.C., unpublished results). These mutants were transformed with a multicopy plasmid library (7), and plasmids that restored growth on raffinose at 33°C were isolated as described in Materials and Methods. In addition to plasmids containing the wild-type *SNF1* gene, four plasmids containing different genes were recovered. One of them, pEL312, is characterized here.

pEL312 suppressed the raffinose nonfermenting (Raf⁻) phenotype caused by *snf1-11ts*, *snf1-12ts*, and *snf1-13ts* at 33°C, but not at 37°C, and restored only very weak growth in a *snf1-Δ10* (2) null mutant at 30°C. Assays of secreted invertase showed that pEL312 restored high-level, regulated invertase expression in *snf1-ts* mutants (Table 2). The strains grew considerably less well on raffinose than would be predicted from their invertase activity, suggesting that the multicopy plasmid suppresses the *SUC2* derepression defect but not other *snf1* defects. The plasmid also complemented the growth defect of a *snf4-Δ2* (3) null mutant on raffinose at 30°C, and pEL312 was independently isolated from the library in a direct selection for multicopy suppressors of *snf4* (E.J.A. Hubbard and M.C., unpublished result).

Analysis and sequence of the suppressor gene

To delimit the suppressor gene in pEL312, we subcloned fragments in the multicopy vector YEp24 (8) and tested their ability to suppress the Raf⁻ phenotype of a *snf1-11ts* mutant at 33°C and a *snf4-Δ2* mutant at 30°C (Fig. 1). The results suggested that the gene lies in the 1.5-kb region between the

One possible model is that multiple copies of *MSN1* suppress the temperature sensitive phenotype of a *snf1-ts* mutant by enhancing the activity of the mutant protein kinase. To assess the SNF1 kinase activity in a *snf1-1ts* mutant carrying pEL312 and in a *msn1-Δ1::URA3* mutant, an immune complex kinase assay (2) was used. The SNF1 protein was immunoprecipitated from crude extracts with affinity-purified anti-SNF1 antibody (2), the immunoprecipitate was incubated with [γ - 32 P]ATP in kinase buffer, and the labeled proteins were analyzed by SDS-

polyacrylamide gel electrophoresis and autoradiography. The presence of the plasmid did not increase the low level of kinase activity detected in the parent strain, and the *msn1-Δ1::URA3* mutation did not reduce or alter the pattern of phosphorylated products detected in this assay (data not shown). These results suggest that the *MSN1* product does not function as an activator of the SNF1 protein kinase and raise the possibility that *MSN1* acts downstream of the kinase in affecting *SUC2* expression.

Nuclear localization of *MSN1*-β-galactosidase fusion protein

To facilitate the subcellular localization of the *MSN1* gene product, we constructed a fusion to β-galactosidase. We joined codon 298 of *MSN1* to the *E. coli lacZ* gene, generating the gene fusion *MSN1*(298)-*lacZ* (Fig. 1). This gene fusion on a multicopy plasmid does not complement *snf1-11ts* or *snf4-Δ2*. Wild-type cells carrying the plasmid were stained with anti-β-galactosidase antibody and examined by immunofluorescence microscopy. The fusion protein was localized in the nucleus (Fig. 3). The sequence Gly-Leu-Lys-Arg-Lys-Arg at position 266 is a potential nuclear targeting signal (26).

LexA-*MSN1* fusion protein activates transcription

The location of the *MSN1* product in the nucleus suggested that overexpression of the *MSN1* protein compensates for defects in SNF1 protein kinase activity by directly affecting transcription of *SUC2*. The possibility that the *MSN1* protein itself functions as a transcriptional activator was tested by using the LexA fusion system of Brent and Ptashne (27). We fused the N-terminal 87 codons of the *E. coli lexA* gene, which encode the DNA binding

domain, to codon 39 of *MSN1* (Fig. 1); the fusion is under control of the *ADHI* promoter. We then determined whether the LexA-*MSN1* fusion protein could bind to a *lexA* operator and activate transcription of a nearby promoter present on a target plasmid. The target plasmids were: the control plasmid pLR1Δ1, carrying a *GAL1-lacZ* fusion and a deletion of *UAS_G* (28); plasmid 1840, derived from pLR1Δ1 by inserting a *lexA* operator 5' to the *G-ALI* promoter (16); and pSH18-18, identical to 1840 except containing six *colE1* overlapping *lexA* operators (S.D. Hanes and R. Brent, personal communication). The wild-type strain MCY829 was doubly transformed with the plasmid expressing LexA-*MSN1* and each target plasmid. As a control, the strain was transformed with pSH2-1, which expresses the first 87 residues of LexA in the LexA₍₁₋₈₇₎ protein (16), and each target plasmid. Activation of *GAL1-lacZ* was assessed by assaying β-galactosidase. The LexA-*MSN1* fusion protein stimulated activation 10- and 100- fold from targets containing one and six copies of the *lexA* operator, respectively (Table 3). Its function was dependent on the *MSN1* sequence in the fusion protein, as no stimulation by the LexA₍₁₋₈₇₎ protein was detected. Thus, the LexA-*MSN1* fusion protein is capable of functioning as a transcriptional activator when bound to the *lexA* operator.

Table 3. Gene activation by the LexA-*MSN1* fusion protein

Target Plasmid	Number of <i>lexA</i> Operators	Expressed Protein LexA ₍₁₋₈₇₎	LexA- <i>MS1</i>
pLR1Δ1	0	0.5	0.4
1840	1	0.6	4
pSH18-18	6	0.6	39

Strains were grown in synthetic complete medium lacking histidine and uracil to maintain selection for both plasmids. Values are averages of assays of β-galactosidase activity in four transformants of strain MCY829.

Figure 3. Nuclear localization of *MSN1*-β-galactosidase fusion protein. Immunofluorescence microscopy was performed as described previously (25). Glucose-grown cells of strain MCY1094 carrying the *MSN1*(298)-*lacZ* gene fusion on a multicopy plasmid were stained with mouse monoclonal anti-β-galactosidase antibody (Promega Biotec) and fluorescein isothiocyanate-conjugated F(ab')₂ fragment of sheep antibody to mouse immunoglobulin G (Sigma) (a). To locate nuclei, cells were also stained with 4',6-diamidino phenylindole (b). No staining was detected in cells lacking the gene fusion (not shown). (c) Phase contrast photograph.

Figure 4. DNA-binding activity of in vitro synthesized *MSN1* protein. DNA binding assays (18) were carried out as described in the text. Binding reactions contained in vitro synthesized *MSN1* protein (+) or protein from a mock in vitro translation carried out without addition of exogenous RNA (-). Reactions contained no plasmid DNA (a); pUC19 DNA with the *SUC2* sequence -381 to -542 (b), -26 to -166 (c), -166 to -390 (d), or -28 to -420 (e); or pUC19 DNA and 10 mg of yeast protein extract prepared as described previously (31) (f). The arrow marks the position of unbound protein.

DNA-binding activity of MSN1

In a simple model, the MSN1 protein might activate *SUC2* transcription by binding to the *SUC2* 5' region. However, conventional gel mobility shift experiments (29, 30) revealed no difference in the pattern of DNA-protein complexes formed with the *SUC2* regulatory region (nucleotides -542 to -381) in wild-type and *msn1-Δ1::URA3* mutant extracts (data not shown). We therefore employed the more sensitive method of Hope and Struhl (18) to test the DNA-binding activity of the MSN1 protein. ³⁵S-labeled MSN1 protein, lacking only the N-terminal 39 amino acids, was synthesized in vitro, and a major protein of the expected size was produced. Plasmids containing the *SUC2* 5' sequences -26 to -166, -166 to -390, -381 to -542, and -28 to -420 cloned in pUC19 were digested to release the inserted *SUC2* DNA, and the DNA was incubated in binding reactions containing the labeled MSN1 protein. The reactions were fractionated by electrophoresis, and in all cases the addition of DNA shifted the mobility of the labeled protein (Fig. 4). Addition of yeast protein extracts to the binding reactions did not significantly alter the pattern (data not shown). In control experiments, incubation of the protein with vector DNA alone also produced the major shifted band (Fig. 4, lane f). It is possible that some minor bands may correspond to complexes formed with the *SUC2* DNA.

The in vitro synthesized MSN1 protein was also incubated with a ³²P-labeled DNA fragment containing the *SUC2* sequence -28 to -420, and the reactions were examined for complexes resulting in gel mobility shift of the labeled DNA. As a control, binding reactions were performed with the products of a mock translation reaction carried out with no added RNA. Only a very faint band was detected corresponding to a complex that was formed only in the presence of the MSN1 protein (data not shown).

Thus, the in vitro synthesized MSN1 protein has DNA-binding activity, but specificity for *SUC2* sequences could not be demonstrated.

DISCUSSION

We report here the isolation and characterization of *MSN1*, a gene that in multicopy restores high-level, regulated invertase expression in a *snf1-ts* or *snf4* deletion mutant. Overexpression of the MSN1 protein could compensate for defects in SNF1 protein kinase activity by a variety of mechanisms. The MSN1 protein does not appear to be an activator of the SNF1 kinase; whether it is a target of the kinase remains to be determined. The ability of the LexA-MSN1 fusion protein to activate transcription when bound to a *lexA* operator suggests that the native MSN1 protein may function as a transcriptional activator. This idea is supported by the nuclear location of the protein and the DNA-binding activity of the in vitro synthesized MSN1 protein. However, we cannot exclude the possibility that the MSN1 sequence fortuitously provides an activation domain in this assay.

Does MSN1 activate *SUC2* expression by a direct mechanism? Although MSN1 has DNA-binding activity, no specificity for *SUC2* sequences was apparent. It remains possible that the native protein binds to *SUC2* 5' sequences in vivo; perhaps some post-translational modification is required that is not present in the in vitro synthesized protein. Alternatively, MSN1 could affect *SUC2* expression indirectly. For example,

the MSN1 protein may bind to and activate expression of another gene that in turn encodes an activator of *SUC2*.

The basic C terminus of the MSN1 protein appears to be required for function. A multicopy plasmid carrying the *MSN1*(298)-*lacZ* fusion, which lacks the C-terminal 84 residues, did not suppress *snf1-ts* or *snf4*. The missing basic region may provide the DNA binding domain.

Disruption of the *MSN1* gene reduced derepression of invertase only a fewfold; in contrast, increased gene dosage dramatically elevated invertase expression in *snf1-ts* and *snf4* mutants. One possible explanation for this apparent discrepancy is that other genes function redundantly with *MSN1* in affecting *SUC2* expression. Perhaps the multiple RNAs detected with *MSN1* probes encode partially homologous, functionally redundant proteins.

The *MSN1* gene apparently affects expression of other genes besides *SUC2*. A multicopy plasmid carrying the same gene, called *FUP1*, was isolated in a selection for improved growth on iron-deficient medium (32).

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