Two Homologous Zinc Finger Genes Identified by Multicopy Suppression in a SNF1 Protein Kinase Mutant of *Saccharomyces cerevisiae*

FRANCISCO ESTRUCH^{1,2} AND MARIAN CARLSON^{1*}

Department of Genetics and Development and Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York, New York 10032,¹ and Departamento de Bioquímica y Biología Molecular, Facultades de Ciencias, Universitat de València, Valencia, Spain²

Received 29 December 1992/Returned for modification 2 February 1993/Accepted 13 April 1993

The *MSN2* gene was selected as a multicopy suppressor in a temperature-sensitive SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *MSN2* encodes a Cys₂His₂ zinc finger protein related to the yeast MIG1 repressor and to mammalian early growth response and Wilms' tumor zinc finger proteins. Deletion of *MSN2* caused no phenotype. A second similar zinc finger gene, *MSN4*, was isolated, and deletion of both genes caused phenotypic defects related to carbon utilization. Overexpression of the zinc finger regions was deleterious to growth. LexA-MSN2 and LexA-MSN4 fusion proteins functioned as strong transcriptional activators when bound to DNA. Functional roles of this zinc finger protein family are discussed.

In the budding yeast *Saccharomyces cerevisiae*, the SNF1 (CAT1/CCR1) protein-serine/threonine kinase is required for expression of many genes in response to glucose limitation (6, 48). This important signalling pathway is as yet incompletely understood, although a number of proteins that function in the SNF1 pathway have been identified. At least two proteins are physically associated with SNF1 in a multiprotein complex: SNF4 (CAT3), which serves as a positive effector (7, 8, 16, 49), and SIP1, which is phosphorylated in vitro (59). The SNF1 pathway appears to inhibit transcriptional repression mediated by the SSN6, TUP1, and MIG1 proteins (26, 33, 34, 50, 58), as mutations in *SSN6*, *TUP1*, and *MIG1* suppress the requirement for SNF1 (35, 56). Further understanding of the regulatory mechanism requires the identification of other components of the SNF1 pathway.

To identify proteins that are functionally related to SNF1, we have sought genes that in increased dosage compensate for defects in SNF1 protein kinase function caused by temperature-sensitive *snf1* (*snf1-ts*) or *snf4Δ* mutations. Such multicopy suppressors are expected to include genes encoding proteins with various functional relationships to SNF1, such as regulatory proteins or targets of the kinase, activators of related kinase pathways, or repressors of an antagonistic pathway. Using this approach, we previously recovered *SIP1*, which encodes a protein that interacts physically with SNF1 and appears to serve as a substrate (59); *MSN1*, which encodes a DNA-binding protein that activates transcription (15); two genes in the cyclic AMP-dependent protein kinase pathway (22); and the *YCK1* casein kinase I gene (39).

Here, we report the selection of a new multicopy plasmid that restores the ability of a *snf1-ts* mutant to grow on raffinose. The suppressor gene, designated *MSN2*, encodes a zinc finger protein of the Cys₂His₂ type first found in the *Xenopus* transcription factor TFIIIA (31). Disruption of *MSN2* caused no phenotype, suggesting the presence of a functionally redundant gene. A second, similar zinc finger

gene was isolated by sequence homology, and deletion of both genes caused phenotypes related to carbon utilization. These two genes, along with *MIG1* (34) and *RGMI* (13), encode a family of yeast proteins related to the mammalian early growth response and Wilms' tumor locus zinc finger proteins (4, 10, 18, 24, 27, 30, 54).

MATERIALS AND METHODS

Strains and genetic methods. Strains of *S. cerevisiae* used in this study are listed in Table 1. Standard methods were used for genetic analysis and transformation (42). Growth on different carbon sources was scored by spotting cell suspensions or by streaking cells on solid medium. Anaerobic growth on raffinose and galactose was scored by incubating plates in GasPaks (BBL).

Isolation of temperature-sensitive alleles of *SNF1*. The centromere-containing plasmid pCE101 carrying the *SNF1* gene on a *XhoI*-*Bam*HI fragment (7) was mutagenized with hydroxylamine (40) and used to transform MCY1845 (*snf1-Δ10*). Ura⁺ transformants (~20,000) were replicated to supplemented synthetic (SD) medium lacking uracil and containing 2% raffinose (SR-Ura). The plates were incubated at 37°C, and 219 transformants that were unable to grow were retested for growth on SR-Ura at 25, 30, and 37°C. Ten showed a temperature-sensitive (Ts) phenotype, and plasmid DNA recovered from five of them conferred the Ts phenotype. The fragment containing the *SNF1* gene was recloned in YCp50 (41) and used to transform the *snf1* mutant. Four of the reconstructed plasmids conferred a Ts phenotype. For these four, the *Clal*-*NruI* *SNF1* fragment was transferred to the integrative vector pRS304 (52) between the *Clal* and *SmaI* sites, and the resulting plasmid was used to transform MCY2119. Integration was targeted to the *snf1-Δ10* locus by cutting with *PstI*. The resulting transformants were tested for anaerobic growth on raffinose and galactose and showed reduced growth relative to wild type at 33°C and no growth at 37°C. The mutations are designated *snf1-11ts* through *snf1-14ts*.

Isolation of the *MSN2* gene. Strain MCY2120 carrying the *snf1-11ts* allele was transformed with a yeast genomic library

* Corresponding author.

TABLE 1. *S. cerevisiae* strains used in this study

Strain ^a	Genotype
MCY730	<i>MATa ade2-101 ura3-52 lys2-801 his3-Δ200 trp1-Δ1 SUC2</i>
MCY731	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 SUC2</i>
MCY1093	<i>MATa his4-539 ura3-52 lys2-801 SUC2</i>
MCY1094	<i>MATa ura3-52 ade2-101 SUC2</i>
MCY1845	<i>MATa snf1-Δ10 ade2-101 ura3-52 SUC2</i>
MCY1853	<i>MATa snf4-Δ2 his4-539 ura3-52 lys2-801 SUC2</i>
MCY2119	<i>MATa ade2-101 trp1-Δ1 his3-Δ200 ura3-52 trp1-Δ1 SUC2</i>
MCY2120	<i>MATa snf1-Δ10::snf1-11ts::TRP1 ade2-101 his3-Δ200 ura3-52 trp1-Δ1 SUC2</i>
W303-1A	<i>MATa ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112</i>

^a All MCY strains are related to S288C. W303-1A was obtained from R. Rothstein (55).

in the multicopy vector YEp24 (5). Transformants were selected on SR-Ura plus 0.1% glucose and incubated anaerobically at 33°C. Plasmid DNAs from *Raf*⁺ transformants were recovered by passage through bacteria, and plasmids containing the *SNF1* gene were identified by restriction analysis. Plasmid pEL32 was recovered and retested by transforming the *snf1-11ts* strain. Four other plasmids were recovered in a slightly different selection described previously (15).

Plasmids containing *MSN2* sequences. pEY32H, -G, -B, -E, and -F contain the indicated sequences cloned in YEp24 (Fig. 1). P1 contains the *SalI*-*EcoRI* fragment of pEY32B cloned in pUC19 (60); the *SalI* site in pEY32B is in the vector. P2 contains the *EcoRI* fragment cloned in pUC19. pEB32 contains the *SalI* fragment cloned in pUC19. To construct pt32-1::URA3 and pt32-2::URA3, the 1.1-kb *URA3* fragment was inserted at the *XhoI* site in plasmids P2 and P1, respectively. To construct pt32-ΔXB::HIS3, the *XhoI*-*BglII* fragment from pEB32 was replaced with the *XhoI*-*BamHI* *HIS3* fragment. pIVT2 contains the *EcoRI* fragment that is in P2 cloned in Bluescript vector pKS (Stratagene); the ATG codon at 492 is used for the transla-

tional start. pGPD32C contains the same *EcoRI* fragment, blunted with Klenow fragment and cloned into the blunted *BamHI* site of pG-3 (47). pLexA-MSN2 contains the *MSN2* sequence from the *EcoRV* site at codon 43 to the 3' *SalI* site cloned into pSH2-1 (21) between the *BamHI* (blunted) and *SalI* sites.

Isolation of *MSN4*. A yeast genomic library in YEp24 (5) was screened for sequences homologous to *MSN2* by colony hybridization at low stringency (29). About 20,000 bacterial colonies were screened for hybridization to probe P2 (Fig. 1), and plasmid DNA was isolated from positive colonies. Plasmids containing the *MSN2* gene were identified by restriction site and Southern blot analysis. One of the remaining plasmids, pEL45, showed substantially stronger hybridization to P2 than did the others. A second plasmid that hybridized weakly contained the *RGM1* gene (13).

Plasmids containing *MSN4* sequences. To construct pZfh45-1::URA3, the *XbaI*-*EcoRI* fragment was subcloned in pUC19, generating pEB45, and the *URA3* gene was inserted in the *EcoRV* site at position 1467 (see Fig. 4). pZfh45-1::TRP1 is identical except that the *TRP1* gene was inserted. To construct pZfh45-Δ2::URA3, the *XbaI*-*EcoRV* fragment from pEB45 was replaced with the *URA3* gene and the *EcoRI*-*XbaI* fragment was inserted to reconstruct the 5' part of the gene, yielding a deletion of nucleotides 605 to 1467. The zinc finger region of *MSN4* was fused to the glutathione *S*-transferase (GST) gene of plasmid pGEX-3X (Pharmacia) (53) in two steps. The *XbaI*-*EcoRI* fragment of pEL45 was cloned into pKS, and then the *EcoRV* fragment (nucleotide 1467-polylinker) was removed from the resulting plasmid and subcloned in the *SmaI* site of pGEX-3X, yielding pZfh45-GEX3X. pGPD45C was constructed by cloning the *EcoRV* fragment into the *BamHI* site of pG-3, using DNA fragments blunted by treatment with Klenow fragment. The translational start is the ATG codon at position 493. To construct pLexA-MSN4, we first cloned the *HincII*-*EcoRI* fragment in pBluescript. We then excised an *NciI* fragment extending from a site at codon 33 to the polylinker, blunted this fragment with Klenow enzyme, and cloned it into the blunted *EcoRI* site of pGEX-3X. A *BamHI*-*SalI* fragment was recovered by cleaving polylinker sites and cloned into the cognate sites of pSH2-1.

FIG. 1. Maps of *MSN2* gene and plasmids. Plasmids are described in the text. The bold arrow indicates 5'-to-3' direction of coding region. Vector sequences are not shown. The ability of multicopy plasmids to suppress the raffinose growth defect of *snf4* and *snf1-11ts* mutant strains is indicated. The *XbaI*-*PvuII* and *PvuII*-*SacI* fragments are 1.5 and 3 kb, respectively. Restriction sites: B, *BglII*; P, *PvuII*; R, *EcoRI*; S, *SalI*; Sc, *SacI*; X, *XbaI*; Xh, *XhoI*. Not all *BglII* sites are shown.

Disruption of the chromosomal *MSN2* and *MSN4* loci. The *msn2-1::URA3* and *msn2-2::URA3* mutations were introduced (43) into the diploid MCY1093 × MCY1094, and *msn2-Δ3::HIS3* was introduced into MCY730. The mutation *msn4-1::URA3* was introduced into MCY730, MCY731, and MCY1093, and *msn4-Δ2::URA3* was introduced into MCY731. Strain W303-1A was disrupted with *msn2-Δ3::HIS3* and *msn4-1::TRP1*. Disruption of the chromosomal locus was verified by Southern blot analysis.

Analysis of DNA and RNA. Restriction fragments were cloned into M13mp18 and M13mp19, and the sequence was determined by the method of Sanger et al. (44) with the 17-nucleotide sequencing primer. The second strand was sequenced by using synthetic oligonucleotides as primers. The program TFASTA (37) was used to compare sequences with those in the GenBank data base. Preparation and analysis of poly(A)-containing RNA was as described previously (45).

Gel mobility shift assays. In vitro transcription of pIVT2 was carried out as described previously (51), and RNA was translated in a wheat germ cell-free system (Promega). Expression of the GST-MSN4 fusion protein from pZfh45-GEX3X in *Escherichia coli* and preparation of protein extracts were performed as described previously (25). In vitro-translated protein from pIVT2 or protein extract containing GST-MSN4 expressed in bacteria was incubated with a ³²P-labeled DNA fragment containing *SUC2* sequences –384 to –542 (46) for 20 min at room temperature in binding buffer (50 mM Tris-HCl [pH 7.7], 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 10 μM ZnCl₂, 0.4 mM phenylmethylsulfonyl fluoride, 10% glycerol). The binding reaction mixtures contained 100 pg of end-labeled DNA, 1 μg of poly(dI-dC), and 1 μl of protein sample in a volume of 15 μl. For the competition experiments, affinity-purified (53) GST-MSN4 protein (0.1 μg) was incubated with a ³²P-labeled DNA fragment containing *SUC2* sequences –381 to –650. Binding reactions were carried out as described above except that reaction mixtures contained 200 pg of end-labeled DNA and 50- or 100-fold molar excess unlabeled competitor DNA in addition to poly(dI-dC). Samples were loaded onto a 6% polyacrylamide gel in 1× Tris-borate-EDTA, and electrophoresis was at 200 V for 2.5 h.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *MSN2* and *MSN4* sequences reported in this paper are L08838 and L08839, respectively.

RESULTS

Selection of a multicopy plasmid that suppresses the growth defect of a *snf1-ts* mutant. Plasmid pEL32 was selected as a multicopy suppressor of the *snf1-11ts* mutation as described in Materials and Methods. The plasmid restored anaerobic growth of a *snf1-11ts* mutant on raffinose at 33°C. Assays of secreted invertase activity, which is required for hydrolysis of raffinose, showed that pEL32 elevated the expression of invertase in this mutant (Table 2). pEL32 was found to suppress the raffinose-nonfermenting (Raf[–]) phenotype caused by the *snf1-11ts*, *snf1-12ts*, and *snf1-13ts* mutations at 33°C but not at 37°C, and it restored only very weak growth in the *snf1-Δ10* null mutant at 30°C. The plasmid also complemented the growth defect of a *snf4-Δ2* null mutant (8) on raffinose at 30°C and restored low-level, regulated invertase expression (Table 2). Immune complex kinase assays (7) of *snf1-11ts* and *snf4* mutants carrying the plasmid showed no restoration of SNF1 protein kinase activity (data not shown), suggesting that the suppressor gene acts down-

TABLE 2. Effects of increased *MSN2* and *MSN4* gene dosage on invertase activity

Relevant genotype (plasmid)	Invertase activity ^a	
	Repressed	Derepressed
Wild type	1	350 (±40)
<i>snf1-11ts</i> (YEpl24)	<1	10 (±1)
<i>snf1-11ts</i> (pEL32)	<1	36 (±3)
<i>snf4-Δ2</i> (YEpl24)	<1	<1
<i>snf4-Δ2</i> (pEL32)	<1	18 (±5)
<i>snf4-Δ2</i> (pEL45)	1	8 (±1)

^a Cells were grown in supplemented synthetic medium lacking uracil and containing 2% glucose at 30°C. Derepression was in the same medium containing 0.05% glucose for 3 h. Invertase activity is expressed as micrograms of glucose released per minute per 100 mg (dry weight) of cells. Values represent averages of assays of two or three transformants. Errors are shown in parentheses.

stream of the kinase or in another pathway. The suppressor gene on pEL32 was designated *MSN2* (multicopy suppressor of *snf*). *MSN1* has been described previously (15). In another study, pEL32 was independently isolated from the library as a multicopy suppressor of *snf4-Δ2* (22).

To localize the *MSN2* gene on pEL32, we subcloned fragments in the multicopy vector YEpl24 and tested for suppression of the *snf1-11ts* and *snf4-Δ2* mutations (Fig. 1). To identify the RNA encoded by the functional region, poly(A)-containing RNAs isolated from glucose-repressed and derepressed wild-type cultures were analyzed by Northern (RNA) blot hybridization. Probes P1 and P2 (Fig. 1) hybridized to a 2.7-kb RNA present in both samples (data not shown); this RNA was not detected in an *msn2* disruption mutant (below).

***MSN2* encodes a zinc finger protein.** We determined the nucleotide sequence of the functional region between the *SacI* site and the *EcoRI* site located 3' to the gene (Fig. 1). A single open reading frame of 704 codons was identified (Fig. 2). The predicted *MSN2* protein of 77,813 Da contains two tandem zinc finger motifs near the C terminus. These fingers are of the Cys₂His₂ type first discovered in the *Xenopus* transcription factor TFIIA (31) and are similar to those of the transcription factor Sp1 (25). The finger regions are closely related to those of the yeast MIG1 and RGM1 proteins (13, 34), mammalian early growth response proteins Egr-1/Zif268/Krox-24/NGFI-A and EGR2/Krox-20 (10, 24, 27, 30, 54), and the Wilms' tumor locus protein (4, 18) (Fig. 3).

The *MSN2* protein also has several other noteworthy sequence features. The N terminus is acidic (net charge of –14 in the first 111 residues), and another acidic stretch lies between residues 260 and 279 (net charge of –9). The C terminus is basic and highly charged (net charge of +13 over the last 90 residues with 41 charges). The protein has a high content of serine (16%), threonine (8%), and asparagine (14%).

Genetic analysis of *MSN2*. To determine the phenotype caused by loss of *MSN2* gene function, we introduced two insertion mutations, *msn2-1::URA3* and *msn2-2::URA3* (Fig. 1), into a diploid, replacing the wild-type sequence on one chromosome (see Materials and Methods). Tetrad analysis of the resulting diploids showed no segregation of any defect in aerobic growth on glucose, growth on raffinose in the presence of antimycin A, or anaerobic growth on raffinose. We then constructed the mutation *msn2-Δ3::HIS3*, which lacks most of the coding region. Introduction of this muta-

FIG. 2. Nucleotide sequence and predicted amino acid sequence of the *MSN2* gene. Nucleotides are numbered on the right, and amino acids are numbered on the left. Amino acids are indicated in standard single-letter code. *, termination codon. Zinc finger motifs are underlined.

tion into the haploid strain MCY730 also caused no detectable phenotype or any change in immune complex assays of SNF1 kinase activity (data not shown).

The *MSN2* gene is located on chromosome XVI, as judged by whole chromosome blot hybridization (14), and genetic analysis showed linkage to *GAL4* on the left arm (23).

Isolation of a homologous zinc finger gene. One explanation for the dispensability of the *MSN2* gene is that the genome contains a second, functionally redundant gene. To test this possibility, we screened a yeast genomic library for hybridization to probe P2 (Fig. 1), which contains the zinc finger-encoding region of *MSN2* (nucleotides 1431 to 2249). Plasmid pEL45 was recovered (Fig. 4). To determine whether pEL45 functions as a multicopy suppressor of *snf1-11ts* and *snf4-Δ2*, the plasmid was used to transform mutant strains. Some restoration of growth on SR-Ura was detected, but the suppression phenotype was weaker than that of pEL32. Assays of *snf4* mutants carrying pEL45 revealed partial restoration of invertase expression (Table 2).

FIG. 3. Comparison of zinc fingers. The zinc finger motifs of *MSN2* and *MSN4* are aligned with those of the *S. cerevisiae* MIG1 protein (34) and mammalian Egr-1 proteins (10, 27, 30, 54). Fingers are numbered according to their positions in the primary sequence. Identical and highly conserved (K, R; D, E; S, T) residues that are shared by *MSN2* and *MSN4* and that are found in at least three sequences are shown in reverse contrast. Dots mark conserved Cys and His residues. Arrowheads mark residues likely to determine sequence-specific DNA recognition (36).

FIG. 4. Maps of *MSN4* gene and plasmids. Plasmids are described in the text. The bold arrow indicates 5'-to-3' direction of coding region. Vector sequences are not shown. The *msn4-1::TRP1* mutation is the same as *msn4-1::URA3* except for the marker. The *EcoRI-BglII* fragment is 2 kb. Restriction sites: B, *Bam*HI; H, *Hind*III; Hc, *Hinc*II; R, *Eco*RI; RV, *Eco*RV; X, *Xba*I.

The region of pEL45 with sequence homology to probe P2 was located by Southern blot analysis of restriction fragments, and sequence analysis was initiated at an *EcoRV* site located between two fragments homologous to P2. We identified an open reading frame of 630 codons that could encode a 69,679-Da protein (Fig. 5). The new gene was

designated *MSN4* (*MSN3* has been assigned to another gene).

The C terminus of the predicted *MSN4* protein includes two zinc finger motifs with high similarity to those of *MSN2* (Fig. 3). The zinc finger sequences of these two proteins are more homologous to each other than to the zinc fingers of other proteins. Residues involved in protein-DNA contacts have been identified by analysis of the crystal structure of a Zif268-DNA complex (36), and the corresponding residues of *MSN2* and *MSN4* are identical to one another, suggesting that the two proteins recognize the same binding sites. Overall, the two proteins also show considerable similarity. In addition to 41% identity (Fig. 6), the proteins are similar in size and amino acid composition. Like *MSN2*, *MSN4* has high contents of serine (15%), threonine (8%), and asparagine (11%). Two short regions corresponding to *MSN2* residues 285 to 313 and 485 to 514 are highly conserved with 69 and 71% identity, respectively.

Disruption of *MSN4* and *MSN2*. To disrupt the *MSN4* gene at its chromosomal locus, we introduced the mutation *msn4-1::URA3* (Fig. 4) into the chromosome of several haploid strains. No growth defect on glucose or raffinose was detected, and invertase expression was normal (Table 3). Thus, *MSN4* appears to be dispensable.

FIG. 5. Nucleotide sequence and predicted amino acid sequence of the *MSN4* gene. Nucleotides are numbered on the right, and amino acids are numbered on the left. Amino acids are indicated in standard single-letter code. *, termination codon. Zinc finger motifs are underlined.

FIG. 6. Comparison of predicted MSN2 and MSN4 proteins. Sequences were aligned by using the BESTFIT program of the Genetics Computer Group (University of Wisconsin) sequence analysis package (11). This alignment shows 41% identity and 60% similarity. MSN2 includes an additional 12 residues at the N terminus.

If *MSN2* and *MSN4* are each dispensable because they are functionally redundant, then disruption of both genes together might have a phenotypic effect. We therefore crossed two haploid strains carrying the *msn2-1::URA3* and *msn4-1::URA3* mutations. Tetrad analysis of the doubly heterozygous diploid showed no segregation of any defect in raffinose utilization in 12 tetrads, but segregants showing slow anaerobic growth on galactose were recovered. The segregation patterns were consistent with the notion that these segregants were double mutants, and Southern blot analysis of genomic DNA from two such segregants confirmed the presence of both disruptions. These double mutants were assayed for invertase, and activity was reduced two- to threefold relative to wild-type and single-mutant activities (Table 3). This reduction was not sufficient to cause a growth defect on raffinose.

In a second experiment, the *msn4-Δ2::URA3* mutation was introduced into a disruptant of MCY730 carrying *msn2-Δ3::HIS3*. Two double mutants were backcrossed to wild type, and the diploids were subjected to tetrad analysis. Because the diploids were homozygous for *his3* and *ura3*, spore clones carrying the disruptions could be identified by their His⁺ and/or Ura⁺ phenotype. Sixteen tetrads were

scored for anaerobic growth on raffinose or galactose and for aerobic growth on glucose or glycerol. The single mutants showed no growth defects. The double mutants grew slightly slower on raffinose and galactose, and expression of invertase was threefold reduced relative to the wild-type level (Table 3).

To explore further the possibility of a growth defect on galactose, we also introduced *msn2-Δ3::HIS3* and *msn4-1::TRP1* mutations into W303-1A (55), which grows much more vigorously on galactose than do strains of the S288C genetic background. The double mutant was dramatically defective in anaerobic growth on galactose or aerobic growth in the presence of the respiratory inhibitor antimycin A (Fig. 7). Double and single mutants grew well on control plates containing glucose. These findings support the view that *MSN2* and *MSN4* are functionally related and affect sugar utilization.

Overexpression of zinc finger regions. We next took another approach to assess the role of these zinc finger proteins in *SUC2* (invertase) gene expression. We reasoned that the zinc finger regions of MSN2 and MSN4, when overexpressed, would compete with native zinc finger proteins (MSN2, MSN4, and any homologs) for binding to recognition sites. By this logic, overexpression of the zinc finger regions might affect *SUC2* expression more severely than deletion of MSN2 and MSN4 if other functional homologs exist. We therefore constructed plasmids expressing the C-terminal regions of MSN2 (codon 492 to end) and MSN4 (codon 493 to end) from the strong *GPD* (glyceraldehyde-3-phosphate dehydrogenase) promoter in vector pG-3 (47). These plasmids, termed pGPD32C and pGPD45C, were used to transform a wild-type strain. The transformants grew much more slowly than controls carrying the vector on medium selective for the plasmid, even with glucose as the carbon source (Fig. 8). To assess effects on *SUC2* expression, we assayed fresh transformants for invertase activity after growth in selective synthetic media. Average derepressed values were 174 for the vector pG-3 (two transfor-

TABLE 3. Invertase activity in *msn2* and *msn4* mutants

Relevant genotype	Invertase activity ^a
Wild type	633 ± 21
<i>msn2-1::URA3</i>	535 ± 45
<i>msn2-Δ3::HIS3</i>	580 ± 20
<i>msn4-1::URA3</i>	645 ± 21
<i>msn4-Δ2::URA3</i>	460 ± 40
<i>msn2-1::URA3 msn4-1::URA</i>	250 ± 10
<i>msn2-Δ3::HIS3 msn4-Δ2::URA3</i>	209 ± 12

^a Assayed in derepressed cultures as for Table 2 except that cells were grown in rich medium (YEP). Values represent averages of two to four assays $\pm$ standard errors. Wild-type segregants from the tetrads were assayed. For all strains, values for glucose-repressed cultures were 1 or <1 .

FIG. 7. Impaired growth on galactose for the *msn2 msn4* double mutant. Strains are derivatives of W303-1A (a) and carry mutation *msn2-Δ3::HIS3* (b) or *msn4-1::TRP1* (c) or both *msn2-Δ3::HIS3* and *msn4-1::TRP1* (d). Strains were streaked on rich medium (YEP) containing 2% galactose and antimycin A (1 μg/ml). Plates were incubated at 30°C.

mants); 82 for pGPD32C (five transformants), and 42 for pGPD45C (five transformants); standard errors were <15%. Repressed values were <2 in all cases. Upon subsequent plating, the cultures expressing zinc fingers were found to yield not only slow-growing colonies but also some fast-growing revertants; the latter may have elevated the invertase activity in the cultures. Thus, overexpression of these zinc finger sequences reduces *SUC2* expression and is generally deleterious to growth.

Zinc finger regions bind DNA. The presence of zinc finger motifs in MSN2 and MSN4 strongly suggests that these proteins bind DNA. To confirm that the zinc finger regions confer DNA-binding activity, we synthesized *in vitro*, from plasmid pIVT2, a polypeptide containing the C-terminal 209 amino acids of MSN2, which includes both zinc fingers. Because our genetic data suggested a role for MSN2 and MSN4 in activation of *SUC2* transcription, we tested for binding to the *SUC2* regulatory region (45, 46). In a gel shift assay, this polypeptide bound to a DNA fragment containing *SUC2* sequences -384 to -542 (Fig. 9A). This fragment contains both of the sites known to be recognized by the related zinc finger protein MIG1 (34). We also expressed in bacteria, from pZfh45-GEX3X, a fusion protein containing the C-terminal 140 residues of MSN4 fused to GST. Protein extracts from cells expressing the GST-MSN4 fusion protein showed DNA-binding activity, while no activity was detected in extracts from cells carrying the vector (Fig. 9A).

We also carried out competition experiments to test the specificity of binding (Fig. 9B). For these assays, affinity-purified GST-MSN4 protein and a labeled DNA fragment containing the *SUC2* sequence -381 to -650 were used. The unlabeled DNA fragment effectively competed for binding (lanes 3 and 4). As nonspecific competitor, we used another unlabeled fragment containing *SUC2* nucleotides -29 to -420. This fragment, which includes neither of the identified

FIG. 8. Evidence that overexpression of zinc finger regions inhibits growth. Wild-type strain MCY730 was transformed with vector pG-3 (a), pGPD32C, expressing the C terminus of MSN2 (b), and pGPD45C, expressing the C terminus of MSN4 (c). Transformants were streaked for single colonies on synthetic medium selective for the plasmid and containing 2% glucose (SD-Trp). pGPD32C and pGPD45C similarly impaired growth on medium containing 2% raffinose (not shown).

MIG1 recognition sites (34), was not an effective competitor (lanes 7 and 8). Similar results were obtained in competition experiments using an affinity-purified GST fusion protein containing the MSN2 zinc fingers (data not shown).

LexA-MSN2 and LexA-MSN4 fusion proteins activate transcription. The preceding genetic and biochemical data suggested that the MSN2 and MSN4 proteins might function as transcriptional activators. To test this idea, we used the LexA fusion system of Brent and Ptashne (2). Fusion proteins with the LexA DNA-binding domain (residues 1 to 87) joined to MSN2 or MSN4 were expressed from the *ADHI* promoter as described in Materials and Methods. Each fusion protein was then tested for the ability to activate expression of a *GAL1-lacZ* target gene when bound to a *lexA* operator located 5' to the promoter; the *UAS_G* is deleted from the target gene (21). Wild-type yeast cells were doubly transformed with expression and target plasmids and assayed for β-galactosidase activity (Table 4). Both LexA-MSN2 and LexA-MSN4 were potent transcriptional activators. In control experiments, no activation was observed when the target gene lacked a LexA binding site or when the LexA DNA-binding domain alone was present. Thus, the MSN2 and MSN4 proteins function as activators when bound to DNA.

DISCUSSION

We have here identified two new genes, *MSN2* and *MSN4*, encoding Cys₂His₂ zinc finger proteins. These genes, together with the previously identified *MIG1* and *RGM1* genes, encode a family of yeast proteins related to the mammalian early growth response and Wilms' tumor locus zinc finger proteins. The mammalian proteins are thought to be involved in signal transduction in response to mitogen

FIG. 9. Autoradiograms of gel mobility shift assays. (A) Binding reaction mixtures contained 32 P-labeled *SUC2* DNA (nucleotides -384 to -542) and no added protein (lane 1); mock in vitro translation products (lane 2); in vitro-translated polypeptide containing MSN2 zinc fingers, encoded by pIVT2 (lane 3); GST-MSN4 protein containing MSN4 zinc fingers, expressed in bacteria from pZfh45-GEX3X (lane 4); or bacterially expressed GST from vector (lane 5). (B) Competition experiment. Binding reaction mixtures contained 32 P-labeled *SUC2* DNA (nucleotides -381 to -650) and no protein (lanes 1 and 5) or affinity-purified GST-MSN4 protein (all other lanes). Unlabeled fragment (nucleotides -381 to -650) was added as specific competitor in 50-fold (lane 3) or 100-fold (lane 4) molar excess. An unlabeled DNA fragment containing *SUC2* nucleotides -29 to -420 was added as nonspecific competitor in 50-fold (lane 7) or 100-fold (lane 8) molar excess. All reaction mixtures contained poly(dI-dG). Experiments were performed as described in Materials and Methods.

and differentiation-specific stimuli. The early growth response genes are rapidly induced in response to growth factors, serum, and other signals, and their products are likely to act as DNA-binding transcriptional regulators involved in modulating cell proliferation and differentiation (10, 24, 27, 30, 54). The Wilms' tumor suppressor may act as a transcriptional repressor (28).

The related yeast family also includes proteins with diverse functions. One member, the MIG1 protein, is involved in the cellular response to glucose and is required for glucose repression of *SUC2*, *GAL1*, and *GAL4* gene expression (17, 19, 33, 34). It has been proposed that MIG1 has a role in tethering the SSN6-TUP1 repressor complex to DNA (26). The *RGM1* gene also appears to encode a transcriptional repressor but does not affect *SUC2* expression (13). In contrast, MSN2 and MSN4 probably function as transcriptional activators.

The MSN2 and MSN4 proteins are similar to one another in several respects and overall share 41% identity. Their zinc fingers are extremely similar and are identical at three key residues likely to determine DNA sequence recognition (36), strongly suggesting that the two proteins recognize the same binding sites. In addition, these key residues are also identical in the two RGM1 zinc fingers and in the first finger of MIG1. The binding sites for MIG1, Egr-1, and the Wilms' tumor protein are related GC-rich sequences (9, 33, 38), and MIG1 recognizes sites in the *SUC2* upstream regulatory region (34). At the *SUC2* locus, a mutation (R15) adjacent to the GC-rich motif in one of the MIG1 binding sites restores *SUC2* derepression when other UAS sequences are deleted

TABLE 4. Transcriptional activation by LexA-MSN2 and LexA-MSN4^a

Expressed protein	β -Galactosidase activity ^b for plasmids containing indicated no. of <i>lexA</i> operators	
	0	1
LexA-MSN2	<1	1,420
LexA-MSN4	<1	1,170
LexA ₁₋₈₇	<1	<1

^a Proteins were expressed from plasmids pLexA-MSN2 and pLexA-MSN4 and from the vector pSH2-1, which expresses the LexA DNA-binding domain (21). Target plasmids were pLR1Δ1 (no *lexA* operator) (57) and 1840 (one operator) (21). Transformants of strain MCY730 were grown with selection for both plasmids.

^b Values are averages for three transformants (two transformants for controls). β -Galactosidase was assayed in permeabilized cells (20), and activity is expressed in Miller units (32).

(46); this result is consistent with the idea that a binding site for an activator overlaps the MIG1 site.

Genetic evidence suggests that MSN2 and MSN4 are related in function as well as sequence. Increased dosage of either gene suppresses the invertase defect of *snf1-ts* and *snf4Δ* mutants, and deletion of both genes together causes phenotypes related to carbon utilization. Both genes play a role in activation of *SUC2* gene expression; however, the physiological importance of this role remains unclear because the double mutant was not profoundly defective. One possibility is that the MSN2 and MSN4 proteins contribute modestly to activation of *SUC2* expression, while other, unrelated proteins are primarily responsible for activation. Another possibility is that there is yet a third, functionally redundant zinc finger protein in this family, and together the three proteins play a major role in activation of *SUC2*. Finally, these zinc finger proteins may affect *SUC2* expression indirectly, for example, via effects on another activator.

MSN2 and MSN4 may recognize the same, or overlapping, sequences in the *SUC2* upstream region that are bound by the MIG1 repressor. If so, these proteins might have antagonistic effects to MIG1 on *SUC2* expression. Studies of the related mammalian zinc finger proteins have provided precedents for such a regulatory mechanism. In the murine adenosine deaminase gene promoter, Zif268 and Sp1 have overlapping binding sites, and Zif268 may down-regulate the promoter by competing with Sp1 for binding (1). The Wilms' tumor protein was shown to bind to an Egr-1 site and function as a transcriptional repressor, thereby antagonizing activation mediated by the Egr-1 protein (28). It remains to be determined whether similar antagonistic regulatory mechanisms operate at the *SUC2* locus. In preliminary studies, *msn2 msn4 mig1* triple mutants resembled *mig1* mutants with respect to *SUC2* regulation (35a), but as noted above, other family members that are functionally redundant with MSN2 and MSN4 may exist.

The primary role of the MSN2 and MSN4 zinc finger proteins may be in controlling expression of genes other than *SUC2*. Certainly, the galactose growth defect of double mutants in the W303 genetic background suggests a role in the galactose utilization pathway. Moreover, in *S. cerevisiae* there are numerous examples of transcription factors that are involved in various, apparently unrelated pathways with different levels of participation in each pathway. A particularly relevant example is provided by the *ACE2* and *SWI5* genes, which encode proteins with 37% overall identity and very similar zinc finger domains (3). ACE2 and SWI5 are

transcriptional activators of two different genes, *CTS1* (encoding chitinase) and *HO* (encoding the endonuclease that initiates mating-type switching), respectively. However, overexpression of either *ACE2* or *SWI5* allows activation of the target gene from the other pathway (12). Interestingly, the *ACE2* gene was originally identified by virtue of its effects in multicopy on expression of a gene other than *CTS1* (3). Thus, by analogy, the primary targets of *MSN2* and *MSN4* action may well be genes that we have not yet identified.

Genetic evidence strongly suggests an important role for this zinc finger protein family in controlling gene expression in *S. cerevisiae*, as overexpression of the *MSN2* or *MSN4* DNA-binding region has deleterious effects on growth. Further studies will be required to elucidate the regulatory roles of each of these proteins and their interactions with one another. It seems possible that these yeast proteins have functional, as well as sequence, similarities to their mammalian counterparts, which play critical roles in control of cell proliferation and differentiation.

ACKNOWLEDGMENTS

We thank Jim New for carrying out assays of DNA-binding activity, Mark Johnston for communication of unpublished genetic data, and Jane Albert Hubbard for computer assistance.

This work was supported by Public Health Service grant GM34095 from the National Institute of Health and by an American Cancer Society Faculty Research Award to M.C. and by DGICYT grants PB88-0352 and PB91-0329 from the Spanish Ministry of Education to F.E.

REFERENCES

- Ackerman, S. L., A. G. Minden, G. T. Williams, C. Bobonis, and C. Y. Yeung. 1991. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. *Proc. Natl. Acad. Sci. USA* **88**:7523-7527.
- Brent, R., and M. Ptashne. 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* **43**:729-736.
- Butler, G., and D. J. Thiele. 1991. *ACE2*, an activator of yeast metallothionein expression which is homologous to *SWI5*. *Mol. Cell. Biol.* **11**:476-485.
- Call, K. M., T. Glaser, C. Y. Ito, A. J. Buckler, J. Pelletier, D. A. Haber, E. A. Rose, A. Kral, H. Yeger, W. H. Lewis, C. Jones, and D. E. Housman. 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* **60**:509-520.
- Carlson, M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. *Cell* **28**:145-154.
- Celenza, J. L., and M. Carlson. 1986. A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science* **233**:1175-1180.
- Celenza, J. L., and M. Carlson. 1989. Mutational analysis of the *Saccharomyces cerevisiae* SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. *Mol. Cell. Biol.* **9**:5034-5044.
- Celenza, J. L., F. J. Eng, and M. Carlson. 1989. Molecular analysis of the *SNF4* gene of *Saccharomyces cerevisiae*: evidence for physical association of the SNF4 protein with the SNF1 protein kinase. *Mol. Cell. Biol.* **9**:5045-5054.
- Christy, B., and D. Nathans. 1989. DNA binding site of the growth factor-inducible protein Zif268. *Proc. Natl. Acad. Sci. USA* **86**:8737-8741.
- Christy, B. A., L. F. Lau, and D. Nathans. 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. *Proc. Natl. Acad. Sci. USA* **85**:7857-7861.
- Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
- Dohrmann, P. R., G. Butler, K. Tamai, S. Dorland, J. R. Greene, D. J. Thiele, and D. J. Stillman. 1992. Parallel pathways of gene regulation: homologous regulators *SWI5* and *ACE2* differentially control transcription of *HO* and chitinase. *Genes Dev.* **6**:93-104.
- Estruch, F. 1991. The yeast putative transcriptional repressor RGM1 is a proline-rich zinc finger protein. *Nucleic Acids Res.* **19**:4873-4877.
- Estruch, F. Unpublished data.
- Estruch, F., and M. Carlson. 1990. Increased dosage of the *MSN1* gene restores invertase expression in yeast mutants defective in the SNF1 protein kinase. *Nucleic Acids Res.* **18**:6959-6964.
- Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature (London)* **340**:245-246.
- Flick, J. S., and M. Johnston. 1992. Analysis of URS_G-mediated glucose repression of the *GAL1* promoter of *Saccharomyces cerevisiae*. *Genetics* **130**:295-304.
- Gessler, M., A. Poustka, W. Cavenee, R. L. Neve, S. H. Orkin, and G. A. P. Bruns. 1990. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature (London)* **343**:774-778.
- Griggs, D. W., and M. Johnston. 1991. Regulated expression of the *GAL4* activator gene in yeast provides a sensitive genetic switch for glucose repression. *Proc. Natl. Acad. Sci. USA* **88**:8597-8601.
- Guarente, L. 1983. Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.* **101**:181-191.
- Hanes, S. D., and R. Brent. 1989. DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. *Cell* **57**:1275-1283.
- Hubbard, E. J. A., X. Yang, and M. Carlson. 1992. Relationship of the cAMP-dependent protein kinase pathway to the SNF1 protein kinase and invertase expression in *Saccharomyces cerevisiae*. *Genetics* **130**:71-80.
- Johnston, M. Personal communication.
- Joseph, L. J., M. M. Le Beau, G. A. J. Jamieson, S. Acharya, T. B. Shows, J. D. Rowley, and V. P. Sukhatme. 1988. Molecular cloning, sequencing, and mapping of *EGR2*, a human early growth response gene encoding a protein with "zinc-binding finger" structure. *Proc. Natl. Acad. Sci. USA* **85**:7164-7168.
- Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian. 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. *Cell* **51**:1079-1090.
- Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson. 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell* **68**:709-719.
- Lemaire, P., O. Revelant, R. Bravo, and P. Charnay. 1988. Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. *Proc. Natl. Acad. Sci. USA* **85**:4691-4695.
- Madden, S. L., D. M. Cook, J. F. Morris, A. Gashler, V. P. Sukhatme, and F. J. Rauscher III. 1991. Transcriptional repression mediated by the WT1 Wilms tumor gene product. *Science* **253**:1550-1553.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Milbrandt, J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science* **238**:797-799.
- Miller, J., A. D. McLachlan, and A. Klug. 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *EMBO J.* **4**:1609-1614.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Nehlin, J. O., M. Carlberg, and H. Ronne. 1991. Control of yeast *GAL* genes by MIG1 repressor: a transcriptional cascade in the glucose response. *EMBO J.* **10**:3373-3377.

34. Nehlin, J. O., and H. Ronne. 1990. Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J.* 9:2891-2898.
35. Neugeborn, L., and M. Carlson. 1987. Mutations causing constitutive invertase synthesis in yeast: genetic interactions with *snf* mutations. *Genetics* 115:247-253.
- 35a. New, J. Unpublished data.
36. Pavletich, N. P., and C. O. Pabo. 1991. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* 252:809-817.
37. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* 85:2444-2448.
38. Rauscher, F. J., III, J. F. Morris, O. E. Tournay, D. M. Cook, and T. Curran. 1990. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science* 250:1259-1262.
39. Robinson, L. C., E. J. A. Hubbard, P. R. Graves, A. A. DePaoli-Roach, P. J. Roach, C. Kung, D. W. Haas, C. H. Hagedorn, M. Goebel, M. R. Culbertson, and M. Carlson. 1992. Yeast casein kinase I homologues: an essential gene pair. *Proc. Natl. Acad. Sci. USA* 89:28-32.
40. Rose, M. D., and G. R. Fink. 1987. *KAR1*, a gene required for function of both intranuclear and extranuclear microtubules in yeast. *Cell* 48:1047-1060.
41. Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* 60:237-243.
42. Rose, M. D., F. Winston, and P. Hieter. 1988. Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. Rothstein, R. J. 1983. One-step gene disruption in yeast. *Methods. Enzymol.* 101:202-210.
44. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
45. Sarokin, L., and M. Carlson. 1985. Comparison of two yeast invertase genes: conservation of the upstream regulatory region. *Nucleic Acids Res.* 13:6089-6103.
46. Sarokin, L., and M. Carlson. 1986. Short repeated elements in the upstream regulatory region of the *SUC2* gene of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 6:2324-2333.
47. Schena, M., D. Picard, and K. R. Yamamoto. 1991. Vectors for constitutive and inducible gene expression in yeast. *Methods Enzymol.* 194:389-398.
48. Schuller, H.-J., and K.-D. Entian. 1987. Isolation and expression analysis of two yeast regulatory genes involved in the derepression of glucose-repressible enzymes. *Mol. Gen. Genet.* 209:366-373.
49. Schuller, H.-J., and K.-D. Entian. 1988. Molecular characterization of yeast regulatory gene *CAT3* necessary for glucose derepression and nuclear localization of its product. *Gene* 67:247-257.
50. Schultz, J., and M. Carlson. 1987. Molecular analysis of *SSN6*, a gene functionally related to the *SNF1* protein kinase of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7:3637-3645.
51. Schultz, J., L. Marshall-Carlson, and M. Carlson. 1990. The N-terminal TPR region is the functional domain of *SSN6*, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 10:4744-4756.
52. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19-27.
53. Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* 67:31-40.
54. Sukhatme, V. P., X. Cao, L. C. Chang, C. H. Tsai-Morris, D. Stamenkovich, P. C. P. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, T. Curran, M. M. Le Beau, and E. D. Adamson. 1988. A zinc finger-encoding gene coregulated with *c-fos* during growth and differentiation, and after cellular depolarization. *Cell* 53:37-43.
55. Thomas, B. J., and R. Rothstein. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell* 56:619-630.
56. Vallier, L., and M. Carlson. Unpublished data.
57. West, R. W., Jr., R. R. Yocum, and M. Ptashne. 1984. *Saccharomyces cerevisiae* *GAL1-GAL10* divergent promoter region: location and function of the upstream activating sequence *UAS_G*. *Mol. Cell. Biol.* 4:2467-2478.
58. Williams, F. E., U. Varanasi, and R. J. Trumbly. 1991. The *CYC8* and *TUP1* proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. *Mol. Cell. Biol.* 11:3307-3316.
59. Yang, X., E. J. A. Hubbard, and M. Carlson. 1992. A protein kinase substrate identified by the two-hybrid system. *Science* 257:680-682.
60. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103-119.