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Molecular Analysis of *SSN6*, a Gene Functionally Related to the *SNF1* Protein Kinase of *Saccharomyces cerevisiae*

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Mutations in the *SSN6* gene suppress the invertase derepression defect caused by a lesion in the *SNF1* protein kinase gene. We cloned the *SSN6* gene of *Saccharomyces cerevisiae* and identified its 3.3-kilobase poly(A)-containing RNA. Disruption of the gene caused phenotypes similar to, but more severe than, those caused by missense mutations: high-level constitutivity for invertase, clumpiness, temperature-sensitive growth, α-specific mating defects, and failure of homozygous diploids to sporulate. In contrast, the presence of multiple copies of *SSN6* interfered with derepression of invertase. An *ssn6* mutation was also shown to cause glucose-insensitive expression of a *GAL10-lacZ* fusion and maltase. The mating defects of *MATα ssn6* strains were associated with production of two a-specific products, a-factor and barrier, and reduced levels of α-factor; no deficiency of *MATα2* RNA was detected. We showed that *ssn6* partially restored invertase expression in a *cyr1-2* mutant, although *ssn6* was clearly not epistatic to *cyr1-2*. We also determined the nucleotide sequence of *SSN6*, which is predicted to encode a 107-kilodalton protein with stretches of polyglutamine and poly(glutamine-alanine). Possible functions of the *SSN6* product are discussed.

The *SNF1* gene of *Saccharomyces cerevisiae* encodes a protein kinase that is required for derepression of many glucose-repressible genes, including the *SUC2* (invertase) gene (5, 8). Mutations in the *SSN6* gene have been isolated as suppressors of the invertase derepression defect in a *snf1* mutant (6). The *ssn6* mutations were found to cause high-level constitutive (glucose-insensitive) invertase synthesis in both *snf1* and wild-type (*SNF1*) genetic backgrounds. These findings suggested that *SSN6* is a negative regulator of *SUC2* and may be a substrate of the *SNF1* protein kinase. The pleiotropic phenotypes of *ssn6* mutants indicated that *SSN6* also affects expression of other genes; the mutants exhibited extreme clumpiness, α-specific mating defects, and a failure to sporulate as homozygous diploids (6). Genetic mapping has led to the finding that *ssn6* is allelic to *cyc8*, a mutation that causes overproduction of iso-2-cytochrome *c* (34). Additional *cyc8/ssn6* alleles have recently been isolated by Trumbly (45).

We report here the cloning and molecular analysis of the *SSN6* gene. We constructed null mutations to determine the phenotype of strains lacking *SSN6* function, tested the effects of multiple copies of *SSN6* on *SUC2* expression, and determined the sequence of the gene. We showed that *ssn6* affects expression of two other glucose-repressible genes and also explored the molecular basis of the mating defects of *MATα ssn6* strains.

MATERIALS AND METHODS

Strains and genetic methods. The *S. cerevisiae* strains used in this study are listed in Table 1. The strains were constructed by standard genetic methods (39), and the genotypes of double mutants were confirmed by complementation analysis. Media and methods for scoring markers have been described (28). Yeast transformations were carried out as described previously (19).

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The *cyr1* mutation from strain T42-36C (gift of K. Matsumoto via T. Toda) was introduced into the S288C genetic background by four serial crosses. *cyr1* segregants were identified by complementation for temperature sensitivity for growth and inability to utilize raffinose. Segregants from the final backcross carried *SUC2* as the only *SUC* gene and were assayed for invertase. One such segregant was crossed to an *ssn6* mutant.

The *GAL80* gene was disrupted (33) in an S288C derivative by transformation with a *Hin*dIII fragment bearing the *gal80-D* null allele of Torchia et al. (44; gift of J. Hopper). Gene disruption was confirmed by physical analysis of the DNA. In crosses, *gal80* segregants were identified by mating to *gal80 MEL1* tester strains and testing the diploids with a chromogenic overlay assay for α-galactosidase activity to score for *gal80* (44).

Cloning the *SSN6* gene. The library (4) contained *Sau*3AI partial fragments of genomic DNA from a strain derived from S288C cloned into YEp24 (3). DNA from this library was used to transform MCY475 to uracil prototrophy, and transformants were recovered from the regeneration agar as described previously (4). The transformants were suspended in sterile water, and the cells in several 10-ml aliquots were sedimented at 1 × *g* until most of the clumpy cells had settled (70 min). The supernatant fluid was removed, and Ura^+ transformants were recovered by plating onto selective medium. Twenty-three colonies were tested for anaerobic growth on raffinose, and six were Raf^-. Plasmid DNA was recovered from these Raf^- transformants by passage through *Escherichia coli* HB101 as described previously (7).

Subclones. pNN116-3 was derived from pLN113-3 by deletion of the 2μm *Eco*RI fragment (Fig. 1). pJS5 and pJS6 contain the indicated fragment in pCGS40 (14). pJS7, pJS11, and pJS12 are subclones in pUC18 (29). pJS9 and pJS10 are subclones in YIp5 (3). pJS18 and pJS19 are derivatives of M13mp18 and M13mp19 (29), respectively. pJS8 and pJS20 are subclones in a pUC18 derivative containing *URA3*. pJS21, pJS22, and pJS23 contain the *URA3 Hin*dIII fragment

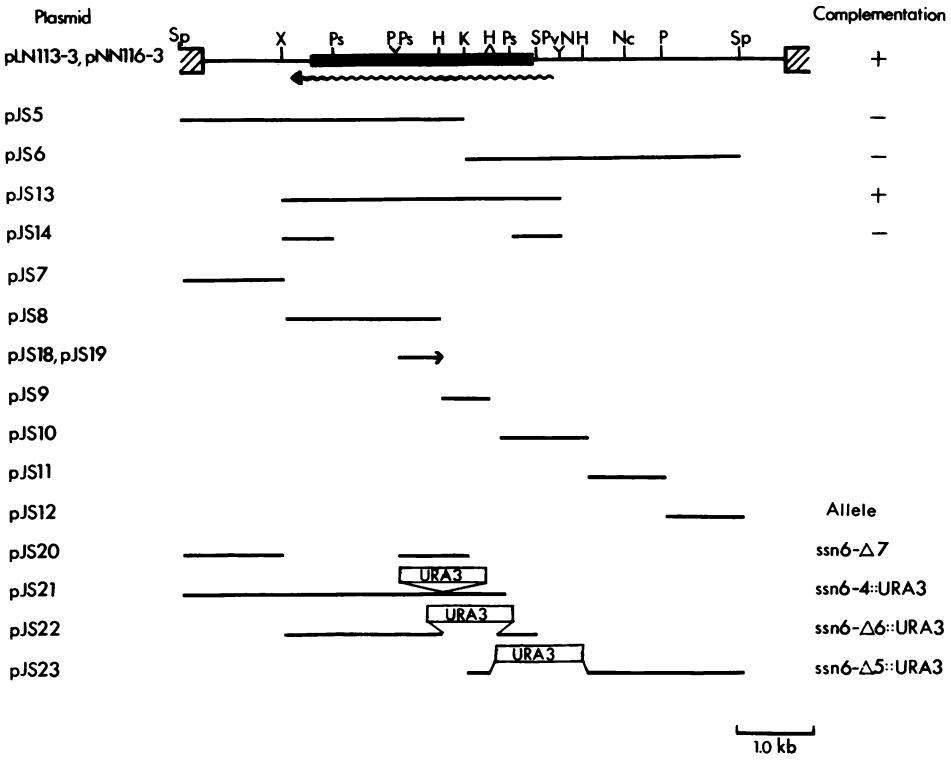

FIG. 1. Restriction maps of *SSN6* clones. Plasmids are described in the text. Only the yeast DNA segment (solid line) is shown, except that the YEp24 vector sequence (hatched box) is indicated in pLN113-3. The wavy arrow indicates the approximate position and direction of transcription of the *SSN6* RNA, and the closed box denotes the open reading frame. Restriction sites within the *URA3* fragment (open box) are not shown. The ability of each episomal plasmid to complement an *ssn6* mutation is indicated. An arrow indicates the 5′-to-3′ direction of the bacteriophage DNA strand of pJS19 that was available for hybridization to RNA in the experiment for which results are shown in Fig. 2; the opposite strand of pJS18 was available. The allele designations of the chromosomal *ssn6* mutations constructed with each plasmid are indicated. Restriction sites: H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; Nc, *Nco*I; P, *Pvu*II; Ps, *Pst*I; Pv, *Pvu*I; S, *Sca*I; Sp, *Sph*I; X, *Xba*I.

inserted between *Hin*dIII sites in the *SSN6* DNA. pJS13 is the *Nhe*I-*Xba*I fragment cloned into the *Xba*I site in pJS30, a derivative of pMH158 (17) constructed by deleting the *Bgl*II-*Bam*HI fragment. pJS14 is pJS13 lacking both *Pst*I fragments from the *SSN6* gene.

Disruption of the *SSN6* locus. Alleles *ssn6-4::URA3*, *ssn6-Δ5::URA3*, and *ssn6-Δ6::URA3* were constructed by the method of Rothstein (33). Plasmids pJS21, pJS22, and pJS23 were digested with restriction enzymes to release a fragment of *S. cerevisiae* DNA carrying the *URA3* insertion, and the digests were used to transform the diploid MCY643 to uracil prototrophy. Transformants in which one copy of the *SSN6* gene was disrupted were identified by Southern blot analysis (40) of genomic DNA. A Ura$^+$ diploid carrying each disruption was subjected to tetrad analysis to recover haploid segregants carrying the disruption. To construct *ssn6-Δ7*, the integrative plasmid pJS20 was used to transform the haploid strain MCY638 and four Ura$^+$ transformants were crossed to strain MCY1094. Ura$^-$ segregants of the resulting diploids were selected on 5-fluoro-orotic acid medium (2). Those segregants in which loss of the integrated plasmid resulted in replacement of the wild-type sequence with the deletion were identified by Southern blot analysis. Two such diploids were subjected to tetrad analysis to recover mutant haploid segregants.

Preparation of DNA and RNA. DNAs were prepared as described previously (7). Poly(A)-containing RNAs were isolated from glucose-repressed or derepressed cultures as described previously (37).

TABLE 1. *S. cerevisiae* strains used

Strain[a]	Genotype	Source
MCY405	*MATα ura3-52 SUC2 gal2*	This laboratory
MCY472	*MATa his4-539 lys2-801 ura3-52 ssn6-1 SUC2*	This laboratory
MCY475	*MATa his4-539 lys2-801 ura3-52 snf1-28 ssn6-1 SUC2*	This laboratory
MCY638	*MATa ura3-52 his4-539 lys2-801 SUC2*	This laboratory
MCY643	*MATa/MATα ura3-52/ura3-52 lys2-801/lys2-801 + /his4-539 SUC2/SUC2*	This laboratory
MCY805	*MATα lys2-801 ssn6-1 SUC2*	This laboratory
MCY1094	*MATα ade2-101 ura3-52 SUC2*	This laboratory
MCY1336	*MATa lys2-801 his4-539 ade2-101 ssn6-4::URA3 (ura3?) SUC2*	This work
MCY1337	*MATα lys2-801 ade2-101 ssn6-4::URA3 (ura3?) SUC2*	This work
T42-36C	*MATa leu1 ura3 trp1 cyr1-2*	K. Matsumoto via T. Toda
X2180-1A	*MATa SUC2 gal2*	YGSC[b]
X2180-1B	*MATα SUC2 gal2*	YGSC
RC634	*MATa sst1-3 ade2 his6 met1 ura1 rme1*	R. Chan
XBH8-2C	*MATα sst2-4 cry1^r his6 ura1 (met?)*	L. Blair
DC17	*MATα his1*	J. Hicks

[a] All MCY strains are isogenic or congenic to S288C.
[b] YGSC, Yeast Genetic Stock Center.

Enzyme assays. Invertase assays were carried out as described previously (7) except that 5 mM EDTA was added to disperse flocculent cultures before the optical density was determined. Cells were grown to the mid-log phase in medium containing 2% glucose. Derepressed cells were prepared by shifting the cells to medium containing 0.05% glucose for 2.5 h at 30°C. Extracellular invertase activity was assayed in whole cells (15) as described previously (7). β-Galactosidase was assayed in permeabilized cells (16); cultures were dispersed with EDTA before the optical density was determined. Maltase was assayed in cells broken by vortexing with glass beads as previously described (28).

Sequence analysis. Restriction fragments were cloned into M13mp18 or M13mp19 (29). The nucleotide sequence was determined by the method of Sanger et al. (35) with the 17-mer sequencing primer and [α-^{35}S]dATP (1) purchased from Amersham Corp.

RESULTS

Cloning the *SSN6* gene. An *ssn6 snf1 ura3* strain (MCY475) was transformed with a genomic library (4) cloned in the multicopy vector YEp24 (3). The parent strain is clumpy and able to utilize raffinose for growth. A plasmid that complements *ssn6* would confer a nonclumpy, Raf$^-$ phenotype. We therefore selected against clumpy transformants and then screened for Raf$^-$ transformants, as described in Materials and Methods. Plasmid DNA was recovered from six such transformants, and five different plasmids were identified. Four plasmids contained overlapping cloned segments, and the smallest of these, pLN113-3, is shown in Fig. 1. Upon retransformation, pLN113-3 complemented the same *ssn6* strain for clumpiness and overproduction of invertase. The plasmid that did not contain an overlapping DNA sequence failed to complement an *ssn6* strain for these phenotypes when retested.

Evidence that pLN113-3 contains the *SSN6* gene was obtained by demonstrating linkage of the cloned DNA to the *SSN6* locus. An integrating plasmid, pNN116-3 (Fig. 1), was derived from pLN113-3 and used to transform MCY638 (*ura3 lys2*). Three Ura$^+$ transformants were mated to MCY405(*ura3*), and the resulting diploids were subjected to tetrad analysis. In 19 tetrads, *lys2* and *ura3* segregated 2:2 in repulsion. These results indicate that in each instance pNN116-3 integrated in the genome of MCY638 at a site tightly linked to *lys2*. Previously, *ssn6/cyc8* has been mapped to a locus 4.5 centimorgans from *lys2* (34). Comparison of the restriction maps of our clones and the previously cloned *LYS2* locus (11) indicated that the 3′ end of the *LYS2* RNA maps about 2 kilobases (kb) from the right edge of the DNA segment cloned in pLN113-3.

To localize the *SSN6* gene within the cloned DNA, we constructed subclones pJS5 and pJS6 (Fig. 1) in the multicopy vector pCGS40 (14) and tested their ability to complement *ssn6* by transforming strain MCY475 (*ssn6-1 snf1-28*). Six Ura$^+$ transformants of each type were examined. All resembled the parent strain in their clumpiness and constitutivity, indicating that neither plasmid complemented the *ssn6* mutation. We then constructed pJS13, which complemented an *ssn6-1* strain for regulation of invertase and all other phenotypes.

Structure and expression of the *SSN6* RNA. To identify the RNA encoded by *SSN6*, poly(A)-containing RNA isolated from glucose-repressed and derepressed cells was analyzed by Northern blot (RNA blot) hybridization. Since neither pJS5 nor pJS6 complemented for *SSN6* function, it seemed likely that the 0.6-kb *Hin*dIII fragment spanning the junction between the two would be homologous to the *SSN6* RNA. This fragment was subcloned in pJS9, and a probe prepared from pJS9 hybridized to a 3.3-kb RNA (Fig. 2). This 3.3-kb RNA was also detected with pJS8 and pJS10 probes, which contain sequences flanking that of pJS9 (Fig. 2). pJS8 and pJS10 also hybridized to other RNAs, most prominently to a 3.7-kb RNA; however, this 3.7-kb RNA does not appear to be derived from the *SSN6* locus because it was present in strains bearing disruptions of the *SSN6* locus (the *ssn6-4::URA3*, *ssn6-Δ5::URA3*, and *ssn6-Δ6::URA3* alleles described below), whereas the 3.3-kb RNA was not present in such mutants (data not shown). These findings in conjunction with the complementation data indicate that the *SSN6* gene encodes the 3.3-kb RNA. A likely explanation for the hybridization to the 3.7-kb and other RNAs was provided by the finding that pJS8 and pJS10, but not pJS9, contain repeated DNA sequences: poly(CAX), where X is A or G, which encodes polyglutamine (see below). pJS11 and pJS12 hybridized to a 2.6-kb RNA, and pJS7 hybridized to a 0.9-kb RNA slightly smaller than the *URA3* RNA; these RNAs are presumably encoded by genes flanking *SSN6*.

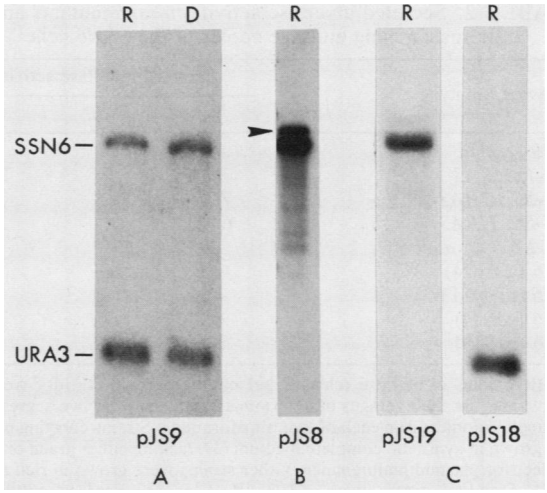

FIG. 2. Structure and expression of the *SSN6* RNA. Poly(A)-containing RNA was prepared from glucose-repressed (R) and derepressed (D) cultures of strain X2180-1B. RNAs were fractionated by electrophoresis through a 1.5% (A) or 1% (B and C) agarose gel containing formaldehyde (23) and transferred to nitrocellulose (43). Size standards were restriction digests of pBR322 DNA. (A) Probe was ^{32}P-labeled pJS9 DNA prepared by nick translation (32). The *URA3* RNA served as an internal standard for the amount of RNA loaded. This blot was later probed with radioactively labeled *SUC2* DNA to confirm that RNAs were prepared from properly glucose-repressed and derepressed cultures. (B) Probe was pJS8 DNA labeled by nick translation. The arrowhead indicates the 3.7-kb RNA. (C) ^{32}P-labeled single-stranded probes were prepared from pJS18 and pJS19 by M13 chain extension (35), omitting chain terminators. The labeled DNA was enzymatically digested with a restriction endonuclease, and the DNA strands were separated by electrophoresis through a denaturing acrylamide gel. The DNA was eluted from the gel as described previously (23). The blot that was probed with JS18 was subsequently probed with YIp5, and detection of the *URA3* RNA confirmed the presence of RNA on the blot.

The direction of transcription of the *SSN6* gene was determined by using single-stranded probes prepared from pJS18 and pJS19, subclones in the vectors M13mp18 and M13mp19 (29). The pJS19 probe hybridized to the 3.3-kb RNA, and the pJS18 probe did not (Fig. 2), indicating that

TABLE 2. Secreted invertase activity in *ssn6* mutants and in strains carrying multiple copies of the *SSN6* gene

Relevant genotype	Secreted invertase activity[a]	
	R	D
SSN6	<1	200
ssn6-4::URA3	765	2,560
ssn6-Δ5::URA3	740	1,970
ssn6-Δ6::URA3	640	2,650
ssn6-Δ7	28	113
SSN6 (YEp24)	<1	210
SSN6 (pLN113-3)	<1	86
SSN6 (pJS14)	<1	224
SSN6 (pJS13)	3	55

[a] Micromoles of glucose released per minute per 100 mg (dry weight) of cells; values are the averages of determinations for at least two segregants or two single colonies from each of two transformants. Strains carrying plasmids were grown in synthetic complete medium (39) lacking either uracil or leucine to select for plasmid maintenance. Other strains were grown in rich medium (YEP). Standard errors were <25%. R, Glucose-repressed cultures; D, derepressed cultures.

the direction of transcription of the *SSN6* gene is as shown in Fig. 1.

The expression of the *SSN6* RNA was not regulated by glucose repression; the RNA was equally abundant in cells grown under conditions of glucose repression or derepression (Fig. 2).

Disruption of the chromosomal *SSN6* locus. To determine the phenotype of a null mutation at the *SSN6* locus, we disrupted the chromosomal copy of the gene by several methods. Deletion and insertion mutations were constructed in plasmids pJS20, pJS21, pJS22, and pJS23 (Fig. 1). These mutations were then introduced into the genome of a diploid strain (*ura3/ura3*) so as to replace the wild-type sequence on one homolog, as described in Materials and Methods. The allele designation of the *ssn6* mutation that was created with each plasmid is indicated in Fig. 1. To determine the phenotype caused by each disruption, a diploid heterozygous for the disruption was subjected to tetrad analysis, and seven or more tetrads were examined. Diploids carrying *ssn6-4::URA3*, *ssn6-Δ5::URA3*, and *ssn6-Δ6::URA3* will be considered first. For each diploid, the *URA3* marker cosegregated 2:2 with the following phenotypes associated with *ssn6* missense mutations: clumpiness, ability to grow on sucrose in the presence of 2-deoxyglucose, and inability to grow on glycerol. We also noted cosegregation of general unhealthiness at 30°C and temperature sensitivity for growth at 37°C, phenotypes which are less marked in an *ssn6-1* mutant. All *MATα* segregants with these phenotypes exhibited a mating-deficient phenotype that was more severe than that of *MATα ssn6-1* mutants. To determine the effects of these mutations on regulation of *SUC2* expression, secreted invertase was assayed in glucose-repressed and derepressed cultures. In each instance, both the constitutive and derepressed activities were much higher than that of the derepressed wild type (Table 2). The three disrupted alleles were recessive with respect to all phenotypes tested: clumpiness, temperature sensitivity, constitutivity for invertase, and inability to utilize glycerol. Each mutation failed to complement *ssn6-1* for these phenotypes, thereby confirming that disruption of the cloned gene created new *ssn6* alleles. This result establishes the identity of the cloned gene as *SSN6*. A diploid with the genotype *ssn6-4::URA3/ssn6-Δ5::URA3* failed to sporulate, as was previously found for diploids homozygous for *ssn6* missense mutations (6).

To determine the phenotype conferred by the *ssn6-Δ7* deletion constructed by using pJS20, two diploids heterozygous for the deletion were subjected to tetrad analysis (11 tetrads of one and 12 of the other). Temperature sensitivity for growth segregated 2:2 in all tetrads and cosegregated with the tightly linked *lys2* marker. Southern blot analysis of genomic DNA from four spore clones of one tetrad showed that the *ssn6-Δ7* deletion cosegregated with temperature sensitivity. Two temperature-sensitive spore clones were assayed for secreted invertase, and both showed low-level constitutive invertase activity and wild-type derepressed activity (Table 2). However, the temperature-sensitive segregants were not clumpy, did not grow on sucrose in the presence of 2-deoxyglucose, exhibited no obvious mating defects, and grew on glycerol. The 0.9-kb RNA was still intact in this deletion mutant, as judged by Northern blot analysis. The *ssn6-Δ7* mutation was found to be recessive to the wild-type *SSN6* allele. It failed to complement *ssn6-4::URA3* for temperature sensitivity and was dominant to *ssn6-4::URA3* for all other phenotypes. Mutants homozygous for *ssn6-Δ7* sporulated normally, yielding tetrads with four spores of the expected phenotype. Sequence analysis (see below) indicated that this deletion removes the carboxy-terminal 371 amino acids of the predicted *SSN6* protein (see Fig. 5).

An *ssn6* null mutation suppresses *snf1*. The *ssn6* missense mutations were originally isolated as suppressors of the sucrose-nonfermenting phenotype caused by *snf1* (6). To determine whether an *ssn6* null mutation would also suppress *snf1*, a diploid heterozygous for both *ssn6-4::URA3* and *snf1-28* was subjected to tetrad analysis. In seven tetrads, temperature sensitivity and clumpiness cosegregated 2:2 with the *URA3* marker linked to *ssn6*, whereas the ability to ferment sucrose and raffinose segregated 3+:1− in six tetrads and 2+:2− in one tetrad. These segregation patterns indicated that *ssn6-4::URA3* suppressed *snf1*. Interactions between this *ssn6* null mutation and other *snf* mutations have been examined (L. Neigeborn and M. Carlson, unpublished results) and were similar to those reported for *ssn6-1* (27).

High *SSN6* dosage prevents full derepression of *SUC2*. Because the phenotype of an *ssn6* mutant suggests that the *SSN6* gene product may act as a repressor, we tested the possibility that multiple copies of the *SSN6* gene would prevent full derepression of the *SUC2* gene. First, derepressed wild-type cells carrying pLN113-3 or the vector YEp24 were assayed for invertase (Table 2). Strains carrying pLN113-3 produced only 40% as much invertase activity as did control strains carrying YEp24. In a second experiment, strains carrying pJS13 and pJS14 were compared. These two plasmids were derived from the high-copy-number vector pMH158 (17) and differ only by a deletion of the *SSN6* coding sequence. Because the *LEU2* gene in pMH158 is poorly expressed, the plasmid must be present in 35 to 100 copies per cell to confer a Leu^+ phenotype (12). Strains carrying pJS13 derepressed only 25% as much invertase activity as did strains carrying pJS14. These data are consistent with the notion that the *SSN6* gene product acts as a negative regulator of the *SUC2* gene.

***SSN6* affects expression of *SUC2* mRNA.** Previously, we showed that an *ssn6* mutation causes constitutive expression of a gene fusion under the control of the *SUC2* upstream region, which suggested that the upstream region mediates the repressive effect of *SSN6* on transcription (36). To confirm that *SSN6* exerts its regulatory effects at the RNA level, expression of *SUC2* mRNA was examined in *ssn6-1* and wild-type cells under glucose-repressing and derepres-

sing conditions. *SUC2* mRNAs were detected by Northern blot analysis of poly(A)-containing RNAs. In an *ssn6* mutant the 1.9-kb *SUC2* mRNA, which is normally glucose repressible, was present at a high level in both glucose-repressed and derepressed cells (data not shown).

ssn6 affects expression of a *GAL10-lacZ* fusion and maltase. Previously, we noted that *snf1* mutants are unable to use galactose as a carbon source (5) and that an *ssn6* mutation does not appreciably suppress this growth defect (6). We next examined the effects of *ssn6* on glucose repression of the *GAL10-lacZ* gene fusion carried on the episomal plasmid pRY123 (46). Both *gal80-D* (null) mutants and wild-type (*GAL80*) strains were tested in case the presence of glucose affected induction by galactose; *gal80* alleviates the requirement for galactose for induction of *GAL10*. Significant β-galactosidase activity was detected in *ssn6 snf1* strains grown in the presence of glucose regardless of the allele at the *GAL80* locus (Table 3). In a *gal80* genetic background, *ssn6* also partially restored the growth of *snf1* mutants on galactose.

We next examined the effects of *ssn6* on expression of the glucose-repressible enzyme maltase. Our strains are derived from the maltose-nonfermenting strain S288C and were therefore transformed with the episomal plasmid pM1.2F (10), which complements the defect. Assays of maltase activity showed that an *ssn6* mutant expressed 25-fold more activity under glucose-repressing conditions (in the presence of maltose) than did the wild type (Table 3). To determine whether *ssn6* suppressed the defect in growth on maltose caused by a *snf1* mutation (5), we carried out tetrad analysis of a diploid heterozygous for both *snf1* and *ssn6* and carrying pM1.2F. The ability to utilize maltose segregated 2:2 in seven tetrads, indicating that *ssn6* does not significantly suppress this growth defect.

a-specific genes are expressed in *MATα ssn6* mutants. Previously, we noted that *MATα ssn6* strains mate less efficiently than the wild type with *MAT***a** tester stains and also mate very weakly with *MATα* tester strains (6). These phenotypes were more striking for the null mutants than for previously isolated missense mutants. To examine the basis for these phenotypes, we tested *ssn6* mutants for synthesis

TABLE 3. Effect of *ssn6* on expression of a *GAL10-lacZ* fusion and maltase[a]

Relevant genotype[b]	β-Galactosidase activity[c]				Maltase activity[d]	
	Glu + Gal	Gal	Glu	Suc	Glu + Mal	Mal
Wild type	<1	690			60	7,700
ssn6	100	550			1,600	8,900
ssn6 snf1	75[e]	370[e]				
snf1	<1					
gal80		540[e]	7	140		
ssn6 gal80		360[e]	140	170		
ssn6 snf1 gal80		310[e]	140	210		
snf1 gal80			4			

[a] Strains were grown in synthetic complete medium (39) lacking uracil (to maintain selection for the plasmid) and containing the indicated sugar at 2%, except that Glu + Mal medium contained 5% glucose. Glu, Glucose; Gal, galactose; Suc, sucrose; Mal, maltose.

[b] Alleles are *ssn6-1*, *snf1-28*, and *gal80-D*.

[c] Units of activity normalized for the optical density at 600 nm of the culture were calculated as described by Miller (26); values are the averages of determinations for two segregants except where noted. Strains carried plasmid pRY123 (46). Standard errors were <30%.

[d] Nanomoles of *p*-nitrophenyl-α-D-glucopyranoside cleaved per minute per milligram of protein. Strains carried plasmid pM1.2F (10). Values are from one assay.

[e] Only one segregant was assayed.

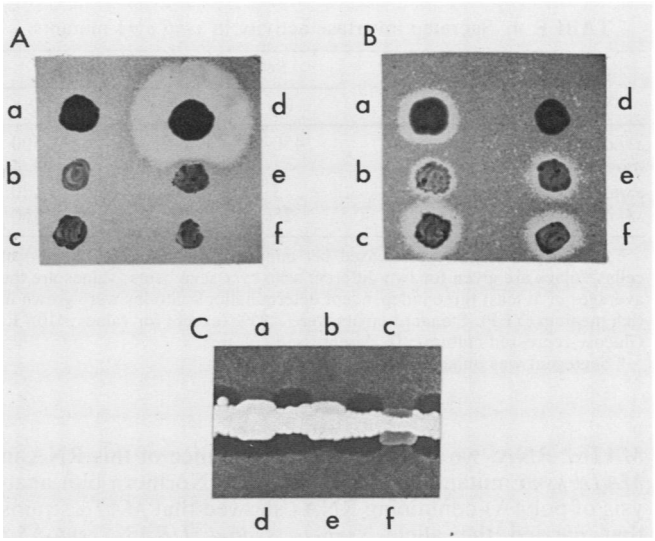

FIG. 3. Bioassays for α-factor, **a**-factor, and barrier. The strains tested were X2180-1A (*MAT***a** *SSN6*) (a), MCY472 (*MAT***a** *ssn6-1*) (b), MCY1336 (*MAT***a** *ssn6-4::URA3*) (c), X2180-1B (*MATα SSN6*) (d), MCY805 (*MATα ssn6-1*) (e), and MCY1337 (*MATα ssn6-4::URA3*) (f). Halo assays were carried out as described by Julius et al. (20). (A) α-factor halo assay. Approximately 4×10^5 cells of strain RC634 (*MAT***a** *sst1*) were spread onto a YEP–2% glucose plate. The strains to be tested for production of α-factor were patched onto the lawn, and the plate was incubated at 30°C for 3 days. (B) **a**-factor halo assay. The conditions and procedures were the same as in panel A except that the tester lawn was XBH8-2C and the plate was incubated at 25°C. (C) Barrier assay. A streak of the α-factor-producing strain DC17 was replica plated onto a freshly spread lawn of strain RC634 (*MAT***a** *sst1*) on a YEP–2% glucose plate, and the strains to be assayed were patched next to the DC17 cells. The production of barrier by the strains tested allowed the lawn to grow in the presence of α-factor. The plate was incubated at 30°C for 3 days.

of α- and **a**-specific gene products. Strains were tested for synthesis of α-factor by the α-factor halo assay (20); in this assay, the production of α-factor by a patch of cells results in a zone of growth inhibition in the surrounding lawn of *MAT***a** *sst1* cells, which are supersensitive to α-factor (9). *MATα ssn6* strains were found to be defective in α-factor activity, eliciting no α-factor halo (Fig. 3A). In an **a**-factor halo assay, *MATα ssn6* mutants produced conspicuous halos on a *MATα sst2* lawn (9), indicating that they produce **a**-factor, a substance normally specific to *MAT***a** strains (Fig. 3B). This finding suggested the possibility that the defect in α-factor activity observed in *MATα ssn6* mutants resulted from abnormal production of barrier, the **a**-specific product of the *SST1* or *BAR1* gene that inactivates α-factor (9, 18, 41). Barrier activity was assayed by patching the strains to be tested next to a wild-type *MATα* strain on a lawn of *MAT***a** *sst1* cells (Fig. 3C). The growth of the *sst1* lawn was inhibited by the α-factor produced by the *MATα SSN6* strain; however, each patch of *MATα ssn6* cells protected the *sst1* lawn from this growth inhibition, indicating that the *ssn6* mutants produced barrier. Production of barrier by the *ssn6* strains could account for the low α-factor activity (41); Northern blot analysis indicated that the *MATα ssn6-4::URA3* strain produced wild-type amounts of α-factor mRNAs.

Previous studies have shown that mutations in the *MATα2* gene cause production of barrier and reduced α-factor activity (41). To determine whether *ssn6* affects expression of the

TABLE 4. Secreted invertase activity in *ssn6 cyr1* mutants

Relevant genotype	Secreted invertase activity[a]	
	R	D
ssn6-1	450	1,500
cyr1-2	<1	4
ssn6-1 cyr1-2[b]	3	120
ssn6-1 cyr1-2	25	460

[a] Micromoles of glucose released per minute per 100 mg (dry weight) of cells. Values are given for two different *ssn6 cyr1* segregants; values are the averages of at least three independent determinations. Strains were grown in rich medium (YEP). Standard errors were <25% (except for values <10). R, Glucose-repressed cultures; D, derepressed cultures.

[b] Segregant was unhealthy.

MATα2 RNA, we compared the abundance of this RNA in *MATα ssn6* mutants and the wild type. Northern blot analysis of poly(A)-containing RNAs showed that *MATα* strains that carried the allele *ssn6-1*, *ssn6-4::URA3*, *ssn6-Δ5::URA3*, or *ssn6-Δ6::URA3* contained the same amount of *MATα2* RNA as did the wild type (data not shown).

ssn6 mutation partially suppresses the invertase defect caused by *cyr1*. Matsumoto et al. reported that a temperature-sensitive mutation in the adenylate cyclase gene (24), *cyr1-2*, caused defects in derepression of invertase, α-glucosidase, and repressible acid phosphatase at temperatures permissive for growth (25). Levels of adenylate cyclase and cyclic AMP were also reduced. Matsumoto et al. suggested that cyclic AMP is required for expression of these enzymes but is unlikely to play a regulatory role since acid phosphatase, unlike the other two enzymes, is not glucose repressible. The role of cyclic AMP in invertase expression remained unclear. To determine whether *ssn6* suppresses the invertase defect caused by *cyr1-2*, we constructed double mutants (see Materials and Methods). We were unable to recover *ssn6-4::URA3 cyr1-2* segregants from 31 four-spored asci from a diploid heterozygous for both mutations. We recovered only four double mutants from 39 four-spored asci from a diploid heterozygous for *ssn6-1* and *cyr1-2*, and two were extremely unhealthy. The other two *ssn6-1 cyr1-2* segregants, one of which was somewhat unhealthy, were assayed for secreted invertase activity (Table 4). The *ssn6* mutation partially suppressed the invertase defect caused by *cyr1-2*, but the double mutants did not exhibit the high-level constitutivity characteristic of the *ssn6* parent. The double mutants were temperature sensitive for growth and were significantly less clumpy than the *ssn6* parent. Thus, *ssn6* was not epistatic to *cyr1-2*. The *SSN6* and *CYR1* genes seem to have antagonistic effects on *SUC2* expression. In a previous study, *ssn6* showed similar interactions with the mutations *snf2*, *snf5*, and *snf6* with respect to *SUC2* expression and the clumpy phenotype (27).

Sequence of the *SSN6* gene. The nucleotide sequence of the *SSN6* gene was determined by the strategy shown in Fig. 4. The nucleotide sequence and the predicted amino acid sequence are shown in Fig. 5. There is an open reading frame of 2,898 base pairs that could encode a 107,177-dalton protein. The position of the coding sequence is consistent with the location of the RNA indicated in Fig. 1.

The most unusual features of the predicted *SSN6* protein are the long stretches of polyglutamine and poly(glutamine-alanine). A block of 16 tandem glutamines is located near the amino terminus (residues 15 to 30), and a second block of 31 glutamines is found at residues 557 to 587. The 64-amino-acid stretch between positions 493 and 556 consists of alternating glutamine and alanine, interrupted only by one lysine and one histidine.

DISCUSSION

We cloned the *SSN6* gene and disrupted the chromosomal locus. The null mutations conferred phenotypes similar to, but more severe than, those conferred by previously isolated missense mutations. An *ssn6* null mutation was an effective suppressor of *snf1* with respect to restoration of *SUC2* expression, and *ssn6* was shown to affect expression of *SUC2* mRNA. We showed that *ssn6* also causes glucose-insensitive expression of a *GAL10-lacZ* gene fusion and the maltase gene. Our studies also provided some understanding of the molecular basis of the effects of *ssn6* on mating competence, a property that is not obviously related to glucose repression. *MATα ssn6* cells produce both **a**-factor and barrier and show reduced levels of α-factor but no deficiency in *MATα2* mRNA.

The nucleotide sequence of *SSN6* proved unenlightening with regard to the function of the *SSN6* gene product. The predicted 107-kilodalton *SSN6* protein was not obviously a DNA-binding protein, and a search of a large data base revealed no significant homology to any other protein except those containing polyglutamine. The most interesting features of the *SSN6* protein are the polyglutamine and poly(glutamine-alanine) stretches, but their significance is not known. Stretches of polyglutamine have been found in the proteins encoded by the *HAP2* (30) and *ANP1* (L. Melnick and F. Sherman, personal communication) genes of *S. cerevisiae*. Repeated sequences that could encode polyglutamine have been found in several *Drosophila* homeotic genes, including Notch (47), engrailed (13, 31), and Antennapedia (21). Another noteworthy feature is that deletion *ssn6-Δ7* removed a substantial portion of the protein, 371 amino acids from the carboxy terminus, without causing a severely mutant phenotype.

We can imagine several possible functions for the *SSN6* product that are consistent with the mutant phenotypes. The simplest possibility is that the *SSN6* protein is a target (direct or indirect) of the *SNF1* protein kinase and that the *SSN6* protein functions as a negative regulator of *SUC2* and other genes; moreover, we cannot exclude the very simple model that *SSN6* encodes a DNA-binding protein. This model satisfactorily accounts for the phenotypes of *ssn6* mutants, including glucose-resistant gene expression, and the epistasis of *ssn6* to *snf1*.

A second possibility is that *SSN6* encodes a phosphoprotein phosphatase that acts antagonistically to the *SNF1*

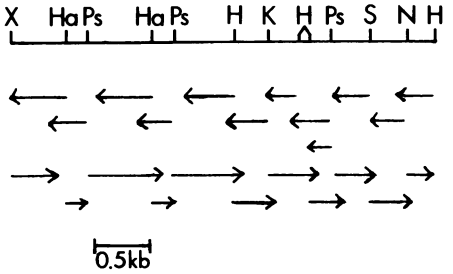

FIG. 4. Sequencing strategy. The arrows indicate the direction and extent of the sequence determined. Regions which were sequenced on only one strand were sequenced independently at least three times, and 94% of the sequence was determined for both strands. Restriction sites: Ha, *Hae*III; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; Ps, *Pst*I; S, *Sca*I; X, *Xba*I.

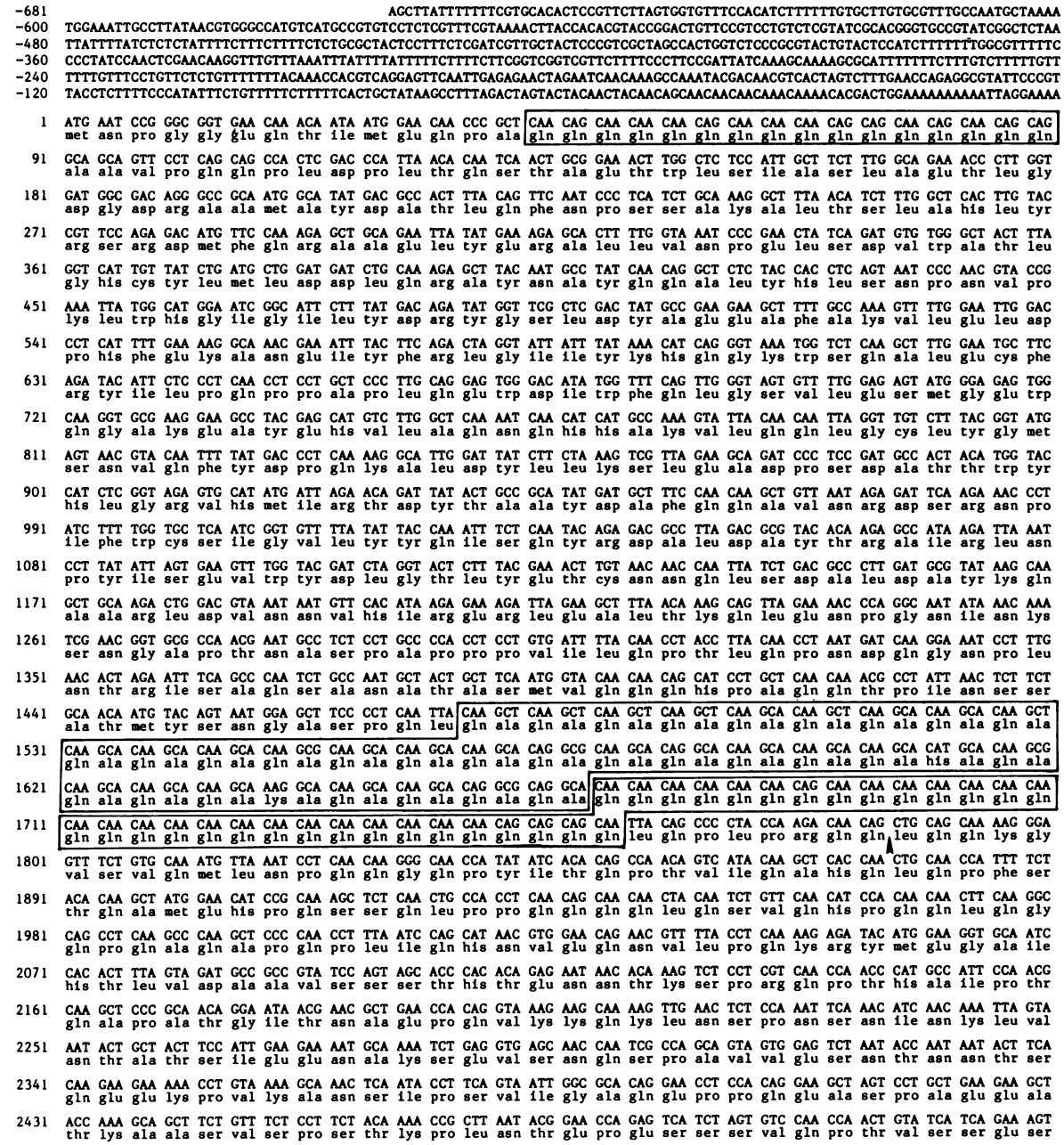

FIG. 5. Nucleotide sequence of *SSN6* gene and predicted amino acid sequence of gene product. Stretches of glutamine and alternating glutamine-alanine residues are boxed. An arrowhead marks the position at which deletion *ssn6*-Δ*7* begins. Asterisks indicate the termination codon. Nucleotides are numbered on the left, and amino acids are numbered on the right.

protein kinase, thereby playing a negative role in glucose repression and perhaps even directly mediating regulatory signals. In an *ssn6* mutant, increased protein phosphorylation could account for the observed phenotypes. It is harder to explain the epistasis of *ssn6* to *snf1*, however, and this model is tenable only if it is additionally postulated that some targets of the *SNF1* protein kinase are also targets of another protein kinase. This second kinase must be sufficiently potent in its activity toward a target of the *SNF1* protein kinase to allow high-level invertase expression in a *snf1 ssn6* mutant but nonetheless must not effectively substitute for the *SNF1* protein kinase in a *snf1 SSN6* genetic background.

A third possible model is that an *ssn6* mutation affects the function of a protein kinase other than that encoded by *SNF1*, either releasing it from its normal regulation or altering its target specificity. It must be imagined that this protein kinase now phosphorylates one or more of the substrates of the *SNF1* protein kinase, thereby bypassing the requirement for *SNF1* function and causing gene expression that is no longer regulated by glucose repression. Other phenotypes of *ssn6* mutants that are not related to glucose repression would, according to this model, also result from altered patterns of protein phosphorylation.

We believe that the gene dosage studies favor a direct role for *SSN6* in control of *SUC2* expression. High *SSN6* gene dosage, and presumably overexpression of the *SSN6* protein, resulted in exactly the phenotype predicted for overproduction of a negative regulator: reduced expression of *SUC2*. Thus, deletion of the gene and increased gene dosage had opposite effects. If *ssn6* were merely a bypass mutation and the wild-type *SSN6* protein were not involved in control of *SUC2* expression, it is difficult to envision how this could occur; for example, it is difficult to reconcile the third model with these data. In contrast, this result is predicted by the first two models.

Further studies are in progress to identify the *SSN6* gene product and determine its function. In this regard, it will be of interest to determine whether an *ssn6* mutation suppresses defects resulting from mutations in protein kinase genes other than *SNF1*.

It is worth noting that genetic evidence suggests that *SSN6* is closely related in function to a gene called variously *TUP1*, *FLK1*, *CYC9*, and *UMR7*. Mutations at this locus cause a similar spectrum of pleiotropic effects, including constitutivity for glucose-repressible enzymes, clumpiness, elevated levels of iso-2-cytochrome *c*, mating and sporulation defects, and other abnormalities (22, 34, 38, 42, 48). We have found that the production of barrier and a-factor in *MATα tup1* mutants (22) is not the result of a deficiency of *MATα2* RNA (unpublished results), as was also true for *MATα ssn6* mutants. Finally, *tup1* resembles *ssn6* in its genetic interactions with *snf* mutations (28). A multicopy plasmid carrying the *SSN6* gene was tested for complementation of a *tup1* mutation but failed to complement (unpublished results). It seems likely that the *SSN6* and *TUP1* gene products are involved in the same regulatory circuit or are otherwise functionally related.

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