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The N-Terminal TPR Region Is the Functional Domain of SSN6, a Nuclear Phosphoprotein of *Saccharomyces cerevisiae*

JANET SCHULTZ, LINDA MARSHALL-CARLSON, AND MARIAN CARLSON*

Department of Genetics and Development and Institute of Cancer Research, Columbia University College of Physicians and Surgeons, New York, New York 10032

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The SSN6 protein functions as a negative regulator of a variety of genes in *Saccharomyces cerevisiae* and is required for normal growth, mating, and sporulation. It is a member of a family defined by a repeated amino acid sequence, the TPR (tetratricopeptide repeat) motif. Here, we have used specific antibody to identify and characterize the SSN6 protein. Both SSN6 and a bifunctional SSN6-β-galactosidase fusion protein were localized in the nucleus by immunofluorescence staining. The N-terminal one-third of the protein containing the TPR units was identified as the region that is important for SSN6 function. Analysis of four nonsense alleles, isolated as intragenic suppressors of an *ssn6::URA3* insertion, revealed that polypeptides truncated after TPR unit 7 provide SSN6 function. Deletion analysis suggested that TPR units are required but that 4 of the 10 TPR units are sufficient. In addition, deletion studies indicated that three very long, homogeneous tracts of polyglutamine and poly(glutamine-alanine) are dispensable. Previous genetic evidence suggested the SSN6 protein as a possible target of the SNF1 protein kinase. Here, we show that the C terminus of SSN6 is phosphorylated in vivo and that the SNF1 kinase is not responsible for most of the phosphorylation. Finally, SSN6 has a modest effect on the maintenance of minichromosomes.

The *Saccharomyces cerevisiae SSN6* gene encodes a protein that functions as a negative regulator of gene expression with a broad range of action and that is required for normal growth. Mutations at the locus cause diverse pleiotropic phenotypes, suggesting that *SSN6* affects the expression of many genes (6, 52, 63). The *ssn6* mutants show slow growth at 30°C, temperature sensitivity for growth, extreme clumpiness, defects in utilization of glycerol, and high-level, glucose-insensitive expression of *SUC2* and other glucose-repressible genes. *MATα ssn6* strains exhibit mating defects because of failure to repress genes that are normally expressed only in *MATa* strains. Homozygous mutant diploids are defective in sporulation. In addition, *ssn6* is allelic to *cyc8*, which causes overproduction of iso-2-cytochrome *c* (49). *SSN6* affects *SUC2* (invertase) gene expression at the transcriptional level, and overexpression of the *SSN6* gene prevents full derepression of *SUC2*, which is consistent with a role for the SSN6 protein as a negative regulator of *SUC2* (52). Taken together, this evidence indicates that SSN6 has a role in regulating expression of genes with a variety of functions and that SSN6 is important for normal cell growth, mating, and sporulation.

Sequence analysis of the *SSN6* gene predicted a 107-kilodalton (kDa) product with several unusual structural features (52, 64). Near its N terminus, the SSN6 protein includes 10 tandem repeats of a 34-amino-acid sequence, termed the TPR (tetratricopeptide repeat) motif, which was recently identified by Sikorski et al. (54). This repeated sequence defines a family of six genes, from several organisms, that encode structurally similar proteins. In addition to *SSN6*, the family includes the *SKI3* gene of *Saccharomyces cerevisiae*, which represses the replication of double-stranded RNA viruses (45), and four genes that function in mitosis: *CDC16* and *CDC23* of *S. cerevisiae* (44), *nuc2*$^+$ of *Schizosaccharomyces pombe* (19), and *bimA* of *Aspergillus nidulans* (36). Hirano et al. (20) proposed that the TPR units form a novel secondary structure, termed a snap helix, in which α-helical segments are associated via "knob and hole" structures and could take on a coiled-coil conformation. Here, we present genetic evidence that the TPR units are important for the function of SSN6.

A striking feature of the predicted SSN6 amino acid sequence is the presence of long homogeneous tracts of polyglutamine and poly(glutamine-alanine). Interestingly, similar tracts are found at similar locations in the protein encoded by *GAL11/SPT13*, which also has a predicted size of 107 kDa (12, 60). Polyglutamine and poly(glutamine-alanine) sequences are present in a variety of other proteins, many of them involved in transcription; for example, yeast HAP2 (41); *Drosophila* Notch, Antennapedia, engrailed, and zeste (13, 29, 42, 43, 65); and rat glucocorticoid receptor (35). The functional significance of these tracts is unclear. We have constructed deletions of these regions to assess their importance for SSN6 function.

Mutations in *SSN6* have been isolated as suppressors that bypass the requirement of having the SNF1 protein kinase for *SUC2* gene expression (6). SNF1 function is required for derepression of a variety of glucose-repressible genes in response to glucose limitation (7). The *ssn6 snf1* double mutants resemble *ssn6* mutants in their high-level constitutive expression of *SUC2*. This genetic evidence suggests that SSN6 is functionally related to the SNF1 protein kinase and points to SSN6 as a candidate for a possible target of the kinase.

In this work, we have used specific antisera to identify and characterize the SSN6 protein. We show by immunofluorescence microscopy that both the SSN6 protein and a bifunctional SSN6-β-galactosidase fusion protein are exclusively localized in the nucleus. We report mutational analyses of the gene that identify the N-terminal third of the protein as the functionally important domain. To address the possibility that the SSN6 protein is a target of the SNF1 protein kinase, we have examined the phosphorylation of the SSN6

* Corresponding author.

TABLE 1. *S. cerevisiae* strains[a]

Strain	Genotype
MCY1093	*MATa his4-539 lys2-801 ura3-52 SUC2*
MCY1094	*MATα ade2-101 ura3-52 SUC2*
MCY1265	*MATa ssn6-4::URA3 his4-539 lys2-801 ura3-52 SUC2*
MCY1326	*MATα ssn6-Δ7 lys2-801 ura3-52 SUC2*
MCY1337	*MATα ssn6-4::URA3 ade2-101 lys2-801 ura3-52 SUC2*
MCY1389	*MATa leu2::HIS3 ura3-52 SUC2*
MCY1729	*MATa SSN6-4R312 lys2-801 ura3-52 leu2::HIS3 SUC2*
MCY1736	*MATa SSN6-4R393 ade2-101 lys2-801 ura3-52 leu2::HIS3 SUC2*
MCY1740	*MATα SSN6-4R309 ade2-101 lys2-801 ura3-52 leu2::HIS3 SUC2*
MCY1751	MCY1093 × MCY1094
MCY1760	*MATα SSN6-Δ8 his4-539 lys2-801 ura3-52 SUC2*
MCY1800	*MATα SSN6-4R402 ade2-101 lys2-801 ura3-52 leu2::HIS3 SUC2*
MCY1801	*MATα ssn6-Δ9 ade2-101 lys2-801 ura3-52 SUC2*
MCY1826	*MATa snf1-K84R his4-539 ade2-101 ura3-52 SUC2*
MCY1832	*MATα SSN6-Δ10 ade2-101 ura3-52 SUC2*
MCY2058	*MATα SSN6-4R309ΔC ade2-101 ura3-52 SUC2*
MCRY750	*MATα ssn6-Δ9 ade2-101 lys2-801 ura3-52::pJSΔ11 SUC2*
RC634[b]	*MATa sst1-3 ade2 his6 met1 ura1 rme1*

[a] All MCY strains are congenic to S288C.
[b] Obtained from R. Chan.

protein in vivo in both wild-type and *snf1* mutant strains. Finally, the effect of *SSN6* on the maintenance of minichromosomes has been examined.

MATERIALS AND METHODS

Strains and general genetic methods. Strains of *S. cerevisiae* are listed in Table 1. Genetic analysis and transformation were carried out by standard procedures (22, 53). Media and methods for scoring markers have been described previously (5).

Preparation of antibody. To construct a *trpE-SSN6* fusion, the 0.6-kilobase (kb) *Hin*dIII fragment from *SSN6* was cloned into pAC351 (gift of E. Abrams), a derivative of pATH3 (T. J. Koerner, J. E. Hill, A. M. Myers, and A. Tzagoloff, Methods Enzymol., in press) containing a *Hin*dIII site in the appropriate frame. The resulting plasmid, pAC0.6-9, encodes a fusion protein of the expected size, 59 kDa. To purify the fusion protein, tryptophan-starved bacterial cells carrying the plasmid were induced for *trpE* expression (58), the insoluble protein fraction was prepared essentially as described (26), and proteins were subjected to preparative electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide (7.5%). The fusion protein was visualized with cold 0.25 M KCl, excised from the gel, and electroeluted (2).

Antibody was raised in two New Zealand White male rabbits by standard procedures (15). SSN6-specific antibody was affinity purified by using the TrpE-SSN6 fusion protein by a modification (8) of the method of Lillie and Brown (31). All experiments shown were carried out with antibody from the same rabbit.

Metabolic labeling of yeast cells. To prepare ^{35}S-labeled cells, cultures were grown 6 to 8 generations, to late log phase, in low sulfate medium (25) containing 100 μM ammonium sulfate, 500 μCi of [^{35}S]sulfate (Amersham Corp.) per ml, and 2% glucose. Cells were collected by centrifugation and washed with cold 5 mM EDTA (pH 8) containing 10 mM sodium azide. Cell pellets were frozen at −80°C.

To prepare ^{32}P-labeled cells, cultures were grown to early log phase in low-phosphate medium containing 150 μM KH_2PO_4 and either glucose or raffinose (2%). Low-phosphate medium contained 0.3% phosphate-depleted yeast extract and 0.5 g of $CaCl_2$, 0.5 g of NaCl, 0.6 g of $MgCl_2$, 1 g of $(NH_4)_2SO_4$, and 20 mg of KH_2PO_4 per liter, adjusted to pH 5.5. Phosphate was precipitated from yeast extract by addition of 35 mM $BaCl_2$ at pH 4.5, and then excess Ba^{2+} was precipitated by addition of 250 mM Na_2CO_3 and heating to 80°C (J. Thorner, personal communication). A portion of the glucose-grown culture was washed and suspended in low-phosphate medium containing 0.15% glucose. [^{32}P]orthophosphate (2.5 mCi; DuPont, NEN Research Products) was then added to 1-ml cultures. The cultures were allowed to undergo two doublings (4 to 4.5 h for the wild type) at 30°C, and the cells were collected by centrifugation. In control experiments to test these conditions, wild-type cells grown in 2% raffinose or shifted to 0.15% glucose derepressed invertase to normal levels. Cells were washed 5 to 10 times in cold 50 mM sodium phosphate (pH 7) containing 10 mM sodium azide. Cell pellets were frozen at −80°C.

Immunoprecipitation and immunoblot analysis. Cell pellets were thawed on ice in 100 μl of phosphate-buffered saline (pH 7) containing 1 mM phenylmethylsulfonyl fluoride, 0.5 mg of ovalbumin (5× recrystallized; Calbiochem) per ml, and 1-octanol or in phosphate-buffered saline containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS (immunoprecipitation buffer). Cells were broken by vortexing with glass beads (0.45 to 0.5 mm) at 4°C. Immunoprecipitation buffer (0.5 ml) was added, and the lysate was cleared by centrifugation. Immunoprecipitation and collection of immune complexes with *Staphylococcus aureus* cells (Boehringer Mannheim) were carried out as described previously (25), except that all volumes were doubled. Samples were then boiled in gel loading buffer (30 mM Tris hydrochloride [pH 6.8], 1.5% SDS, 8% glycerol, 2.5% β-mercaptoethanol) for 1 min, *S. aureus* cells were removed by centrifugation, and samples were loaded on SDS-polyacrylamide gels (28). After electrophoresis, gels containing ^{32}P-labeled proteins were fixed, dried, and exposed to film. Gels containing ^{35}S-labeled proteins were prepared for fluorography as described previously (25).

For immunoblot analysis, proteins were separated by electrophoresis and electroblotted to nitrocellulose as described previously (62), except that methanol was omitted. SSN6 products were detected by using affinity-purified polyclonal rabbit anti-SSN6 antiserum or monoclonal mouse anti-β-galactosidase (Promega Biotec). The primary antibody was detected by using goat anti-rabbit immunoglobulin G (Fc)-alkaline phosphatase conjugate or goat anti-mouse immunoglobulin G (heavy and light chain)-alkaline phosphatase conjugate and the ProtoBlot Immunoblotting System (Promega Biotec).

Synthesis of SSN6 protein in vitro. The *Nhe*I-*Xba*I fragment containing the *SSN6* gene was inserted in front of the T3 promoter in the Bluescript vector pKSM13$^-$ (Stratagene) such that translation of the transcripts would initiate at the first ATG of the *SSN6* coding sequence. The resulting plasmid, pT3S6, was digested with *Sac*I, which cleaves 3′ to *SSN6*. The DNA (2.5 μg) was treated with proteinase K and used as a template for in vitro transcription with T3 RNA polymerase (Pharmacia) as described previously (24). About 20 μg of full-length (3.7 kb) RNA was synthesized. RNA (0.5 or 2 μg) was incubated in cell-free translation reactions

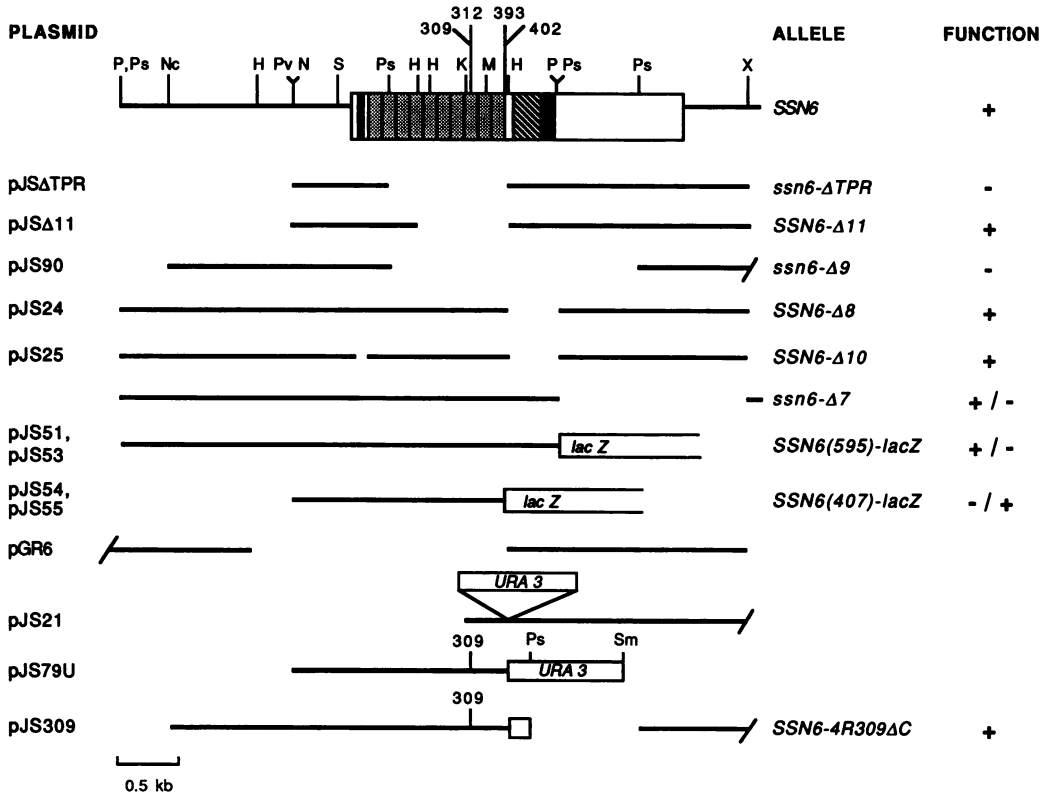

FIG. 1. Structure of *SSN6* gene and restriction maps of plasmids. The open box denotes the *SSN6* coding sequence. The TPR repeats (▨), polyglutamine (■), and poly(glutamine-alanine) (▧) are shown. Plasmids are described in the text. pJS90, pJS21, and pJS309 extend 0.8 kb 3′ to the *Xba*I site. pGR6 extends to an *Sph*I site located 1 kb 5′ to the *Pvu*II site. Vector sequences are not shown. Allele designations are indicated. The *ssn6-4::URA3* allele bears the insertion present in pJS21 (52). Positions of nonsense mutations are marked. *URA3* is transcribed in the same direction as *SSN6*, from left to right. Restriction sites: H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Nhe*I; Nc, *Nco*I; P, *Pvu*II; Ps, *Pst*I; Pv, *Pvu*I; S, *Sca*I; Sm, *Sma*I; X, *Xba*I.

in the presence of [^{35}S]methionine. The rabbit reticulocyte (Promega Biotec) and wheat germ (Du Pont, NEN Research Products) systems were used as recommended, except that the latter reaction mixtures contained 100 mM potassium acetate and 2 mM magnesium acetate.

Immunofluorescence microscopy. Cells were grown to mid-log phase in rich medium (YEP) (53) containing 2% glucose and were derepressed by a shift to medium containing 0.05% glucose for 2.5 h. Cells carrying episomal plasmids were grown in synthetic complete medium lacking uracil, and derepression was carried out for 3 h. Cells were prepared and stained as described previously (9), except that incubations with antibody were carried out overnight at 4°C. Cells were stained with 4′,6-diamidino-2-phenylindole and then were observed and photographed as before (9).

Enzyme assays. Glucose-repressed and derepressed cultures were prepared as described above. Flocculent cultures were dispersed by addition of 5 mM EDTA before the optical density was determined. Extracellular invertase activity was assayed in whole cells (14).

Construction of *SSN6-lacZ* gene fusions. Manipulation of DNA was carried out by standard methods (33). pJS51 and pJS53 (Fig. 1) were constructed by cloning the *Pvu*II fragment into the *Sma*I sites of YEp353 and YIp353 (37), respectively, thereby generating the *SSN6(595)-lacZ* gene fusions. To construct pJS54, the *Nhe*I-*Hin*dIII fragment, which contains two internal *Hin*dIII sites, was inserted into YEp353 via polylinkers. The *SSN6(407)-lacZ* gene fusion from pJS54 was transferred to YIp353 to yield pJS55.

Construction of pJSΔ11. The *Nhe*I-*Xba*I fragment was cloned via polylinkers into the *Xho*I and *Xba*I sites of pRS306 (55). The resulting plasmid was digested with *Hin*dIII and recircularized with ligase, generating pJSΔ11.

Construction of pJSΔTPR. pJSΔ11 was digested with *Pst*I, the ends were filled in with T4 DNA polymerase, and the DNA was digested with *Xho*I, which cuts in the vector 0.2 kb 5′ to the *Nhe*I site. The 0.7-kb fragment was gel purified. Separately, pJSΔ11 was digested with *Hin*dIII, the site was filled in with Klenow fragment, and the DNA was digested with *Xho*I. The 6.4-kb fragment was isolated and ligated to the 0.7-kb fragment to construct pJSΔTPR. Two independent constructions were sequenced to verify that the *SSN6* coding sequence remained in frame across the deletion. The DNA from these two plasmids was digested with *Stu*I to target integration to the *ura3* locus in yeast cells.

Construction of the *ssn6-Δ9* mutation. pJS90 is a derivative of YIp5 (4) carrying the *Nco*I-*Sal*I fragment from pNN116-3 (52) with a deletion of the *Pst*I fragment (Fig. 1). pJS90 was digested with *Xba*I and used to transform the diploid MCY1751 to uracil prototrophy. Ura$^-$ segregants were selected (3), and heterozygous diploids were subjected to tetrad analysis. The presence of the *ssn6*-Δ9 allele in haploid segregants was confirmed by blot hybridization analysis (57) of yeast DNA prepared as described previously (21).

Construction of *SSN6-Δ8* and *SSN6-Δ10* alleles. To construct pJS24 (Fig. 1), the *Kpn*I-*Xba*I fragment was cloned into the 1.8-kb *Xho*I-*Eco*RI fragment of pMH158 (17) via polylinker sequences. The plasmid was cleaved at the

*Hin*dIII and *Pvu*II sites, and the ends were filled in with Klenow fragment and ligated. Next, the *Pvu*II-*Kpn*I fragment containing the 5′ half of *SSN6* was inserted, and the *URA3* gene was inserted into the *Xba*I site.

To delete the N-terminal polyglutamine tract, the 2.6-kb *Pvu*II-*Kpn*I fragment of pJS24 was cloned into M13mp19 (39). Oligonucleotide-directed mutagenesis was carried out by using the Bio-Rad Muta-Gene M13 in vitro mutagenesis kit and a 32-base oligonucleotide with the sequence 5′-GAACAACCCGCTCAAGCAGCAGTTCCT-3′ (purchased from Research Genetics). Fifteen glutamine codons are deleted between the underlined nucleotides. The mutated fragment was recovered from a recombinant bacteriophage and used to replace its wild-type counterpart in pJS24, thus generating pJS25.

pJS24 and pJS25 were digested with *Kpn*I and *Nhe*I, respectively, and were used to transform MCY1751. Ura$^-$ segregants were selected, heterozygous diploids were subjected to tetrad analysis, and haploid segregants were identified by blot hybridization as above. A haploid strain carrying the *SSN6-Δ10* deletion was also obtained by transforming MCY1094.

Isolation and genetic analysis of revertants. Six single colonies of strain MCY1337 were suspended in 5 mM EDTA to disperse the cells and were spread on YEP–2% glucose plates. Cells were exposed to 30 J of UV radiation per m^2 and incubated in the dark for 6 days at 37°C. Revertants were purified and retested.

To test for dominance, the six revertants were crossed to the *ssn6-4::URA3* mutant MCY1265. The diploids grew at 37°C, were able to utilize glycerol, and were not clumpy, indicating that all six mutations are dominant. The revertants were then crossed to strain MCY1389 (*SSN6 ura3*). Tetrad analysis of the diploids confirmed that the *URA3* marker inserted at the *SSN6* locus was still genetically linked to *lys2*, which is tightly linked to *SSN6*. No segregants with an Ssn6$^-$ phenotype were recovered, indicating that in each case the mutation was linked to the *ssn6-4::URA3* allele. To confirm that the phenotypic reversion was due to a single nuclear mutation, tetrad analysis was carried out on the diploids made by crossing two revertants (*SSN6-4R312* and *SSN6-4R393*) to the *ssn6-4::URA3* mutant; as expected, temperature sensitivity, clumpiness, and failure to grow on glycerol showed 2:2 segregations. The six revertants were then crossed to each other in 10 different pairwise combinations, and tetrad analysis of the diploids confirmed that all of the mutations were at a single genetic locus.

Recovery of revertant alleles from genome and nucleotide sequence analysis. The gap-repair method of Orr-Weaver et al. (40) was used to recover the revertant alleles. Plasmid pGR6 (Fig. 1) carries the *SSN6* gene with a deletion of the internal *Hin*dIII fragments cloned in a derivative of YEp351 (18) lacking the *Hin*dIII site. pGR6 was digested with *Hin*dIII, and the linear DNA was gel-purified and used to transform *leu2* segregants derived from crosses between revertants and wild type. Gap-repaired plasmids were recovered from transformants by passage through bacteria. Most of the clones contained the *URA3* insertion and the 0.6-kb *Hin*dIII fragment but did not extend far enough 5′ to include the *Pst*I or *Sca*I sites. The entire region 5′ to the *URA3* insertion was successfully recovered by integrating a plasmid at the *SSN6-4R309* and *SSN6-4R312* loci and excising with appropriate enzymes. No restriction site alterations were identified in the DNA recovered by this method.

For sequence analysis of the DNA recovered by the gap-repair method, restriction fragments were cloned into M13mp18 (39). The nucleotide sequence was determined by the method of Sanger et al. (50) with the 17-mer sequencing primer and [α-^{35}S]dATP purchased from Amersham Corp. Codons 172 to 407 were sequenced for *SSN6-4R309* and *SSN6-4R402*, codons 172 to 352 were sequenced for *SSN6-4R312*, and codons 352 to 407 were sequenced for *SSN6-4R393*.

Reconstruction of *SSN6-4R309* allele from sequenced DNA. The sequenced *Kpn*I-*Mlu*I fragment carrying the *SSN6-4R309* mutation was used to replace the corresponding fragment in pJS21 (52), which contains the *ssn6-4::URA3* insertion, thereby generating pDC79. pDC79 was also sequenced. The wild-type *Nhe*I-*Kpn*I fragment was then moved into pDC79, yielding p794. The *Nhe*I-*Xba*I fragment from p794 was used to replace (48) the wild-type *SSN6* sequence in strain MCY1094.

Plasmid pJS79U was constructed by cloning the *Kpn*I-*Sma*I fragment from pDC79 into a derivative of pUC19 (67) carrying the *Nhe*I-*Kpn*I fragment from *SSN6*. pJS309 carries the 1.1-kb *Pst*I fragment from pJS79U (containing the *SSN6-4R309* mutation) inserted into the *Pst*I site of an integrative plasmid carrying the same *SSN6* sequence that is present in pJS90. The *URA3* gene was also inserted at the *Xba*I site. pJS309 was digested with *Nhe*I and used to transform MCY1094 and MCY1751. Selection with 5-fluoro-orotic acid was applied, and haploid strains with the *SSN6-4R309ΔC* allele were recovered as described above.

Plasmid stability assay. The method of Maine et al. (32), with modifications, was followed. Transformants were streaked for single colonies, and cultures were grown in synthetic complete medium lacking uracil (SC-ura) (53), except that stability of pRS315 was assayed by using synthetic complete medium lacking leucine throughout the procedure. Cells from each culture were inoculated into YPD (53) at a density of 5×10^4 (experiment A) or 1×10^3 to 3×10^3 (experiment B) cells per ml and grown to stationary phase (about 10 generations for experiment A and 15 generations for experiment B). Samples of each culture were diluted in 5 mM EDTA to disperse the cell clumps present in the *ssn6* mutant cultures and were plated in duplicate onto both YPD and SC-ura plates. The percent stability is expressed as follows: [(number of colonies formed on SC-ura)/(number of colonies formed on YPD)] × 100. For the *ssn6* mutants, the percent stability represents an upper estimate because the presence of small clumps of cells reduced the number of colonies formed on YPD. Small clumps, containing an average of 2.7 cells, were visible under the microscope. In control experiments, cells were inoculated into SC-ura instead of YPD, and in all cases the stability ranged from 70 to 100%.

RESULTS

Identification of the SSN6 protein. To identify the SSN6 protein, we prepared specific antiserum to a TrpE-SSN6 fusion protein encoded by the *Escherichia coli trpE* gene fused in frame to codons 206 to 407 from *SSN6*. The fusion protein was purified from bacteria and used to raise antibody in rabbits. To detect the SSN6 protein, yeast cells were metabolically labeled with [^{35}S]sulfate, and proteins were immunoprecipitated with affinity-purified antibody. The antibody recognized a 135-kDa protein that was present in the wild type, absent from a *ssn6-Δ9* deletion mutant (described below; Fig. 1), and present at elevated levels in a strain carrying the *SSN6* gene on a multicopy plasmid (Fig. 2A,

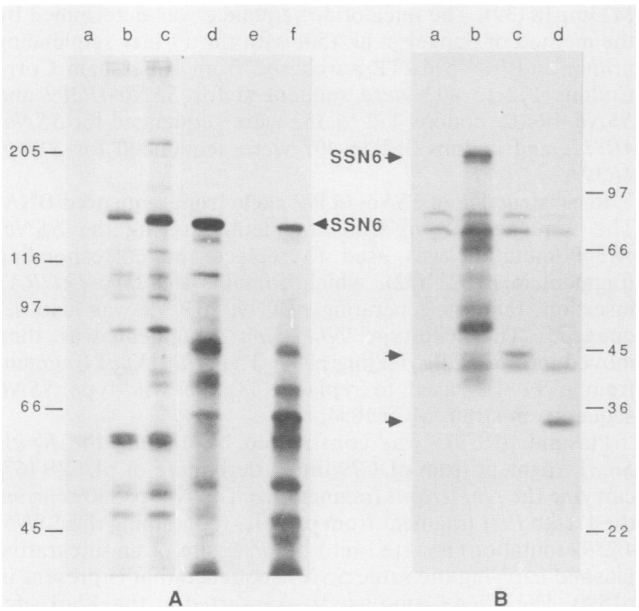

FIG. 2. Identification of SSN6 protein by immunoprecipitation. (A) Proteins immunoprecipitated with affinity-purified rabbit anti-SSN6 antibody, separated by electrophoresis on SDS-polyacrylamide (7.5%), and detected by fluorography. Proteins were prepared from ^{35}S-labeled cells from strains MCY1801 (*ssn6-Δ9*) (lane a), MCY1093 (*SSN6*) (lane b), and MCY1093 carrying *SSN6* on the multicopy plasmid pLN113-3, a derivative of YEp24 (lane c) (53). Proteins were products of in vitro translation of RNA transcribed from pT3S6 (lanes d and f) or no exogenous RNA (lane e) in rabbit reticulocyte (lanes d and e) or wheat germ cell-free translation systems (lane f). The autoradiographic exposure for lanes a through c was 10-fold longer than for lanes d through f. (B) Proteins immunoprecipitated with anti-SSN6 serum and separated by electrophoresis on SDS-polyacrylamide (10%). Proteins were prepared from ^{35}S-labeled cells of strains MCY1801 (*ssn6-Δ9*) (lane a), MCY1093 carrying the multicopy plasmid pLN113-3 (lane b), MCY1800 (*SSN6-4R402*) (lane c), and MCY1740 (*SSN6-4R309*) (lane d). Arrows mark positions of truncated polypeptides. Positions of protein size standards are indicated.

lanes a to c; Fig. 2B, lanes a and b). These results indicate that the antibody recognizes the SSN6 protein.

Immunoblot analysis also revealed a 135-kDa protein that was present in wild type and absent from an *ssn6-Δ9* deletion mutant (data not shown). The protein was present in the same abundance in glucose-repressed and derepressed cells, as is the *SSN6* RNA (52). The SSN6 protein was also detected at approximately wild-type levels in glucose-repressed and derepressed *snf1* mutant cells, indicating that *snf1* does not affect expression of SSN6 (data not shown).

The size predicted for the SSN6 protein from the nucleotide sequence was 107 kDa. The protein was synthesized in vitro (see Materials and Methods) and migrated as a polypeptide of 135 kDa (Fig. 2A, lanes d and f). These findings suggest that modifications of the native yeast protein are unlikely to be responsible for its aberrant migration.

Nuclear localization of SSN6 protein. Genetic evidence suggested that the SSN6 protein functions as a negative regulator of transcription. If SSN6 acts by a direct mechanism to repress gene expression, one would expect the protein to reside in the nucleus. To ascertain the subcellular localization of the protein, wild-type cells were stained with affinity-purified anti-SSN6 antibody and examined by immunofluorescence microscopy. The SSN6 protein was completely localized in the nucleus (Fig. 3), and the staining pattern was the same in both glucose-repressed and derepressed cells (either shifted to low glucose or grown in raffinose) (data not shown). In control experiments, no staining of *ssn6-Δ9* or *ssn6-4::URA3* mutant cells was observed (not shown). The *ssn6-Δ9* allele lacks the coding sequence included in the *trpE-SSN6* gene fusion used to generate antibody and thus provides an appropriate control for nonspecific staining.

Because the TrpE-SSN6 fusion protein used to raise antibody contains TPR units, it was possible that the antibody cross-reacted with structurally similar proteins. It could be argued that the absence of staining in the *ssn6* null mutant reflects effects of the *ssn6* mutation on other proteins, rather than simply the absence of SSN6 itself. We therefore turned to a different method to confirm the nuclear localization of the SSN6 protein: the protein was tagged with β-galactosidase and detected by using anti-β-galactosidase antibody.

Construction of bifunctional *SSN6-lacZ* gene fusions. We sought to construct a gene fusion that provided *SSN6* function in order to ensure that the fusion protein was localized to its normal site of action. Previous studies showed that deletion of the C-terminal sequence in the *ssn6-Δ7* allele (Fig. 1) did not produce a severely mutant phenotype (52) (Table 2). We therefore constructed an in-frame fusion between codon 595 and the *lacZ* gene, designated *SSN6(595)-lacZ* (Fig. 1). Integration of this gene fusion (on pJS53) in single copy in an *ssn6-Δ9* mutant greatly reduced clumpiness and restored growth at 37°C, growth on glycerol, and nearly normal regulation of invertase expression (Table 2), but did not remedy the defect in α-factor production (see Fig. 5). Multiple copies of the fusion (on pJS51) also partially complemented *ssn6*.

A second in-frame gene fusion designated *SSN6(407)-lacZ* was constructed with a fusion point at codon 407. This fusion proved to be only marginally functional when integrated on pJS55 in single copy (Table 2), but the multicopy plasmid pJS54 partially complemented *ssn6* (data not shown).

The two gene fusions encoded proteins of the predicted sizes (160 and 180 kDa), as judged by immunoblot analysis with anti-β-galactosidase antibody (data not shown). Proteins were prepared from the strains used for immunofluorescence studies. The two fusions produced comparable β-galactosidase activity.

Nuclear localization of bifunctional SSN6-β-galactosidase fusion proteins. Wild-type (*SSN6*) cells carrying the bifunctional *SSN6(595)-lacZ* gene fusion integrated in single copy were stained with antibody to β-galactosidase and examined by immunofluorescence microscopy (Fig. 4). The fusion protein was localized in the nucleus in both glucose-repressed (Fig. 4c and d) and derepressed cells (Fig. 4a and b). Cells carrying the gene fusion on a multicopy plasmid showed the same staining pattern (Fig. 4e and f). The *SSN6(407)-lacZ* fusion product was also localized in the nucleus (Fig. 4g and h). Previous studies have shown that β-galactosidase is not targeted to the nucleus unless fused to a nuclear protein (9). These findings confirm that SSN6 is a nuclear protein.

The SSN6 protein contains the sequence Pro-Gln-Val-Lys-Lys-Gln-Lys (residues 732 to 738), which resembles previously identified nuclear localization determinants (for a review, see reference 56). The fusion protein does not include this sequence, but it may be transported to the nucleus in association with the intact SSN6 protein.

Isolation of tightly linked, dominant mutations that sup-

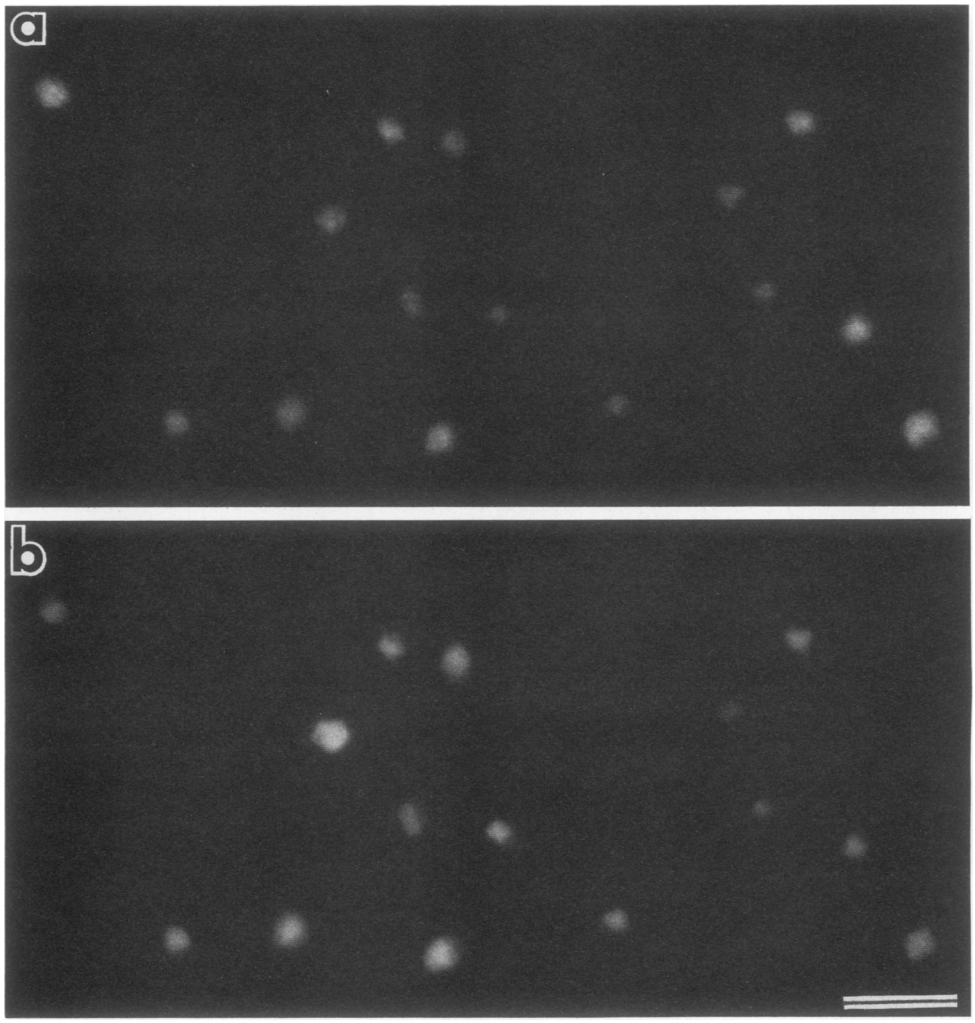

FIG. 3. Nuclear localization of SSN6 protein by immunofluorescence microscopy. Glucose-repressed cells of strain MCY1093 were fixed and stained with affinity-purified anti-SSN6 antibody and then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (a). Cells were also stained with 4',6-diamidino-2-phenylindole to identify nuclei (b). No staining was detected when the primary antibody was omitted. Bar, 5 μm.

press *ssn6-4::URA3*. We attempted to isolate extragenic suppressors of an *ssn6* null mutation to identify other genes that are functionally related to *SSN6*; instead, we recovered intragenic second-site suppressors that identified the N terminus as the important functional domain of the *SSN6* product. We selected revertants of a haploid strain carrying *ssn6-4::URA3* (Fig. 1) (52) by mutagenizing cells and selecting for growth at 37°C. Six independent revertants were characterized. All grew on glycerol, and none was clumpy. All exhibited substantially improved regulation of invertase expression (Table 3), except for one that also grew less well at 37°C. *MAT*α strains carrying the reverted loci were tested for ability to elicit an α-factor halo on a *MAT***a** *sst1* lawn (Fig. 5). Only two resembled the wild type, although all mated normally in standard genetic crossings. These assays indicate that the wild-type phenotype was restored to varying degrees in the different revertants.

Genetic analysis, described in detail in Materials and Methods, showed that all six revertants carried dominant mutations. In each case, a reversion event occurred at a locus tightly linked to *SSN6* and the *URA3* insertion remained at the *SSN6* locus.

TABLE 2. Phenotypes of strains carrying different *SSN6* alleles

Relevant genotype	Plasmid-borne allele	Invertase activity[a]		α-Factor halo[b]
		Repressed	Derepressed	
SSN6		<1	200	+
ssn6-Δ7		28	110[c]	−
SSN6-Δ8		2	200	+
SSN6-Δ10		<1	200	+
ssn6-Δ9		410	1,480	−
ssn6-Δ9 ura3::pJSΔTPR	*ssn6-ΔTPR*	550	1,620	−
ssn6-Δ9 ura3::pJSΔ11	*SSN6-Δ11*	<1	430	+
ssn6-Δ9 ura3::pJS53	*SSN6(595)-lacZ*	57	180	−
ssn6-Δ9 ura3::pJS55	*SSN6(407)-lacZ*	240	860	−

[a] Micromoles of glucose released per minute per 100 mg (dry weight) of cells; values are the averages of determinations for at least two different strains or two assays; standard errors, <25%.

[b] Taken from Fig. 5 and similar experiments. −, Halo not present; +, halo present.

[c] Previous data from Schultz and Carlson (52).

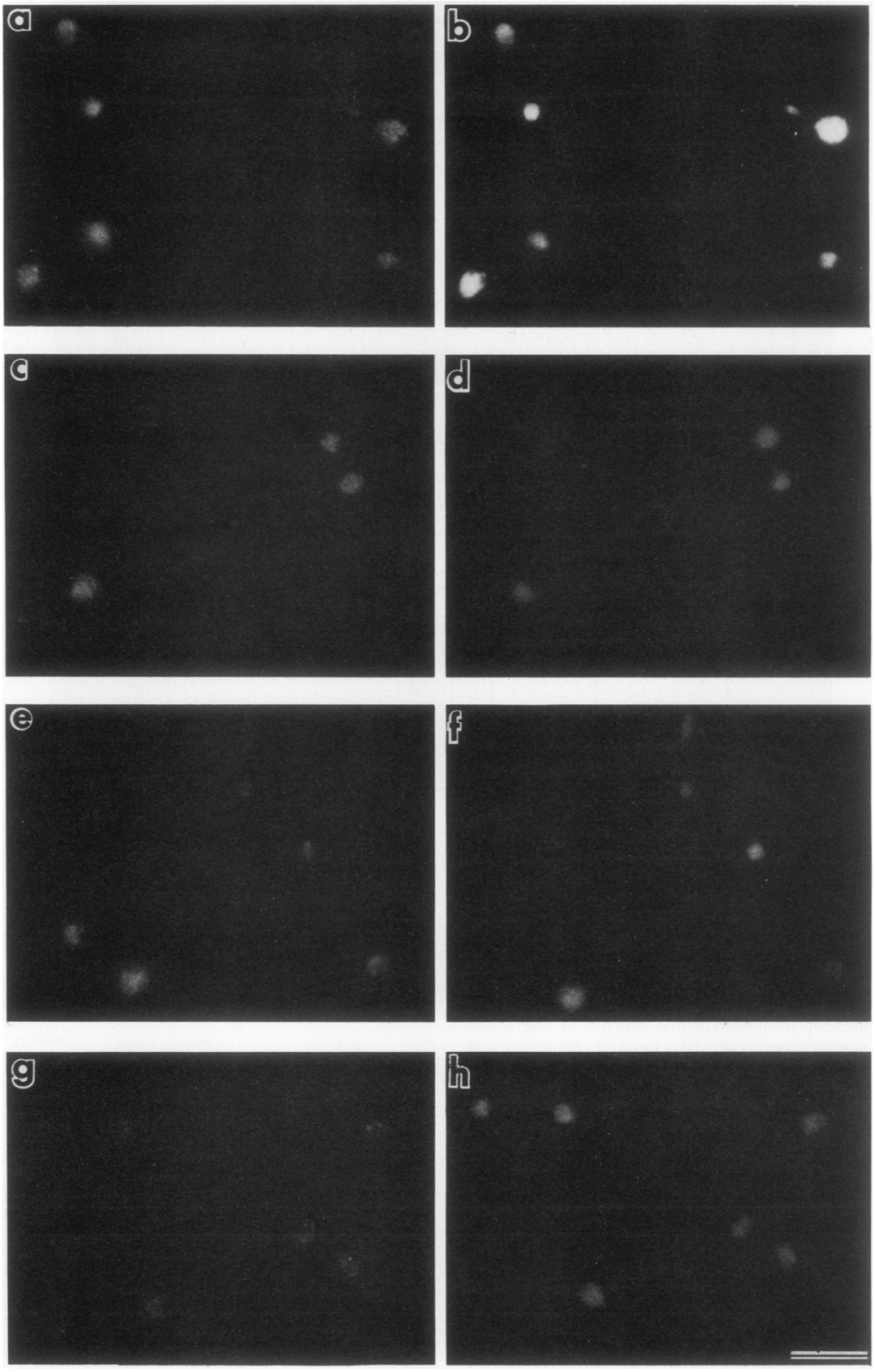
a
b
c
d
e
f
g
h

TABLE 3. Phenotypes of revertants

Relevant genotype	Mutation	Invertase activity[a]		α-Factor halo[b]
		Repressed	Derepressed	
SSN6		<1	200	+
ssn6-4::URA3		310	960	−
SSN6-4R309	AGA→TGA	2	280	+
SSN6-4R312	TAT→TAA	29	250	+
SSN6-4R393	AGA→TGA	39	590	−
SSN6-4R402	AGA→TGA	9	550	−
SSN6-4R8D		12	540	−
SSN6-4R6C		207	1,020	−
SSN6-4R309[c]		2	230	ND
SSN6-4R309ΔC		2	220	ND

[a] Micromoles of glucose released per minute per 100 mg (dry weight) of cells. Values are averages of at least two assays of each revertant or assays of two transformants or two segregants; standard errors, <25%.

[b] Taken from Fig. 5. Halo assays were carried out on *MATα* strains. +, Halo present; −, halo not present. ND, Not determined.

[c] Reconstructed allele.

Nonsense mutations 5′ to the insertion in *ssn6-4::URA3* restore SSN6 function. To determine the molecular basis of these reversion events, we examined genomic DNA. Diagnostic restriction digests revealed no gross rearrangements at the *SSN6* locus. The mutations were recovered from four revertants for sequence analysis (see Materials and Methods). In each case, the introduction of a nonsense mutation 5′ to the *URA3* insertion appeared responsible for reversal of the mutant phenotype (Table 3 and Fig. 1). The sequenced mutations are designated *SSN6-4Rn*, where *n* is the number of the mutated codon. The other two mutations are named according to the assigned number of the revertant.

For the *SSN6-4R309* allele, the revertant locus was reconstructed from sequenced DNA to prove that the nonsense mutation was responsible for the phenotypic reversion. A sequenced fragment carrying the mutation was used to replace the corresponding wild-type sequence from the parent *ssn6-4::URA3* allele (see Materials and Methods). Two strains carrying the reconstructed allele were assayed for secreted invertase activity and yielded values close to those of the original revertant (Table 3). This experiment confirms that the mutation is responsible for the revertant phenotype.

The simple interpretation of these results is that the truncated protein encoded by the nonsense allele is functional. A more complicated possibility is that the C-terminal *SSN6* coding sequence located 3′ to the *URA3* insertion is expressed and is required, in *trans*, for the functioning of the N-terminal polypeptide. To eliminate this possibility, we constructed the *SSN6-4R309ΔC* allele (Fig. 1), which carries the *SSN6-4R309* sequence 5′ to the *Pst*I site in *URA3* but lacks the C-terminal *SSN6* coding sequence (see Materials and Methods). Strains carrying this allele were indistinguishable from the wild type with respect to growth at 37°C, growth on glycerol, lack of clumpiness, and mating properties (Table 3). These experiments indicate that the truncated *SSN6-4R309* product functions in the absence of any C-terminal polypeptide.

Thus, truncation of the SSN6 protein after codon 309, midway through TPR unit 8, results in a protein that is almost fully functional by all criteria considered.

Identification of truncated polypeptides. Strains carrying *SSN6-4R309* and *SSN6-4R402* contained polypeptides of 31 and 44 kDa, respectively, that were detected by immunoprecipitation with anti-SSN6 antibody (Fig. 2B, lanes c and d). These sizes correspond well to the predicted sizes of 34 and 45 kDa and verify the sequencing data.

***SNF1* is required in *SSN6-4R309* strains.** Mutations in *SSN6* were isolated as suppressors of the invertase derepression defect of *snf1* mutants (6). To explore the genetic interaction of various *SSN6* alleles with *snf1*, we constructed double mutants (Table 4). The *ssn6-4::URA3 snf1* strains showed the high-level, constitutive invertase expression characteristic of the *ssn6* parent. In contrast, the *SSN6-4R309* allele did not suppress *snf1*, and the double mutants failed to derepress invertase. Similarly, *ssn6-Δ7*, which confers a nearly wild-type phenotype, did not suppress *snf1*. Thus, *SNF1* is required for invertase expression in mutants producing these truncated SSN6 proteins.

TPR units are required for SSN6 function. To assess the importance of the repeated TPR units, we constructed deletions in the TPR region. The 10 TPR units extend from codon 46 to 398 (54). First, we constructed an in-frame deletion of codons 99 to 406 on pJSΔTPR. Only the first TPR unit remains intact in this deletion, designated *ssn6-ΔTPR*. Two independently constructed versions of pJSΔTPR were integrated at the *ura3* locus of the *ssn6-Δ9* mutant MCY1801. The transformants showed the high-level constitutive invertase expression (Table 2), clumpiness, slow growth, and defect in α-factor production characteristic of *ssn6* mutants, indicating that this allele did not provide SSN6 function.

We next constructed a deletion of codons 173 to 406 on pJSΔ11. The deletion, *SSN6-Δ11*, interrupts the coding sequence within TPR unit 4 but resumes in frame at a region that fortuitously resembles the missing repeat sequence. The deleted protein contains at least three, and probably four, TPR units. pJSΔ11 was integrated in single copy at the *ura3* locus in MCY1801 (*ssn6-Δ9*). The three integrants examined resembled the wild type, except that their derepressed invertase activity was slightly higher (Table 2). Thus, 3 or 4 of the 10 TPR units are sufficient for SSN6 function.

Deletion of most of the *SSN6* coding sequence. A deletion removing codons 101 to 863 of the *SSN6* gene was constructed. The N-terminal sequence, which includes only one intact TPR repeat, was left in frame with the remaining 103 C-terminal codons (Fig. 1). The deletion, designated *ssn6-Δ9*, was introduced into the genome of a diploid strain, and tetrad analysis of two independent heterozygous diploids yielded 2:2 segregations for a phenotype indistinguishable from that of previous null mutants. A high-copy-number

FIG. 4. Nuclear localization of a bifunctional SSN6-β-galactosidase fusion protein. Cells were fixed and stained as described in Materials and Methods. Cells were stained with mouse monoclonal anti-β-galactosidase antibody (Promega Biotec) and then with fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin G antibody (Sigma) (a, c, e, and g). Cells were also stained with 4′,6-diamidino-2-phenylindole to identify nuclei (b, d, f, and h). (a and b) Glucose-derepressed cells of strain MCY1093 carrying pJS53 [*SSN6(595)-lacZ*] integrated in single copy at the *ura3* locus; (c and d) same as panels a and b except that the cells are glucose repressed; (e and f) derepressed cells of MCY1093 carrying *SSN6(595)-lacZ* on multicopy plasmid pJS51; (g and h) derepressed cells of MCY1093 carrying the *SSN6(407)-lacZ* fusion on multicopy plasmid pJS54. No staining was detected in cells lacking the fusion protein, and no staining was detected when anti-β-galactosidase antibody was omitted. Bar, 5 μm.

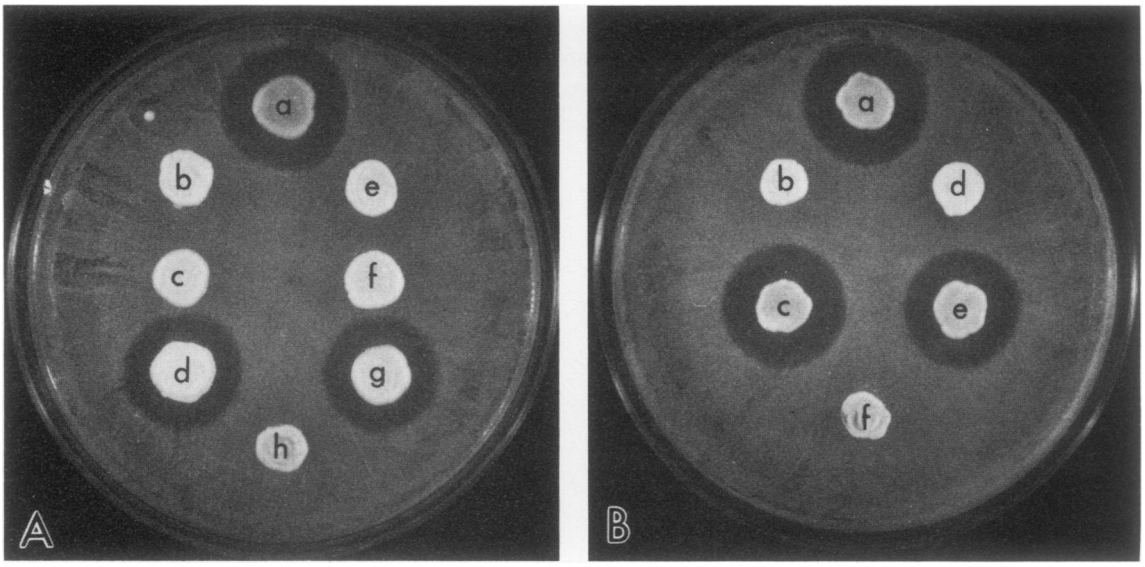

FIG. 5. α-Factor halo assays. Strains to be tested for production of α-factor were patched onto a lawn (5×10^5 cells) of strain RC634 (*MATa sst1*) on a YEP-2% glucose plate, and the plate was incubated at 30°C for 3 days, as described previously (23). The strains tested were *MATα* strains carrying the following *SSN6* alleles: (A) *SSN6* (a), *SSN6-4R6C* (b), *SSN6-4R8D* (c), *SSN6-4R312* (d), *SSN6-4R393* (e), *SSN6-4R402* (f), *SSN6-4R309* (g), *ssn6-4::URA3* (h); (B) *SSN6* (a), *ssn6-Δ7* (b), *SSN6-Δ8* (c), *ssn6-Δ9 ura3::pJS53* [integrated *SSN6(595)-lacZ*] (d), *SSN6-Δ10* (e), *ssn6-Δ9* (f).

plasmid carrying the *ssn6-Δ9* allele provided no detectable *SSN6* function, as assayed by complementation of *ssn6-1*.

Polyglutamine and poly(glutamine-alanine) tracts are dispensable. The *SSN6* sequence encodes strikingly long and homogeneous polyglutamine and poly(glutamine-alanine) tracts (Fig. 1). To determine whether these tracts are important for function when the SSN6 protein is not truncated, we deleted codons 407 to 595, thereby removing the 64-amino-acid stretch of poly(glutamine-alanine) and the 31 tandem glutamines. The coding sequence distal to the deletion remains in frame. The deletion, designated *SSN6-Δ8*, was introduced into the genome of a diploid strain, and two independent diploids heterozygous for the deletion were subjected to tetrad analysis. Haploid segregants carrying the *SSN6-Δ8* allele showed no phenotypic difference from wild-type segregants with respect to α-specific mating defects, regulation of invertase expression, growth at 37°C, growth on glycerol, or clumpiness (Fig. 5 and Table 2).

The *SSN6-Δ8* product still contained a 16-amino-acid polyglutamine tract near the N terminus that might function redundantly with the sequence that was deleted. We therefore used oligonucleotide-directed mutagenesis to remove 15 of the 16 tandem glutamines (amino acids 16 to 30), thereby generating a double deletion designated *SSN6-Δ10*. Tetrad analysis of a heterozygous diploid showed no segregation for the above phenotypes. Two haploid segregants carrying the allele were identified by blot hybridization, and no phenotypic difference from the wild type was discerned (Fig. 5 and Table 2). Thus, these tracts appear to be dispensable for the SSN6 functions that were tested.

SSN6 is a phosphoprotein in vivo. The suppression of *snf1* mutant phenotypes by *ssn6* mutations suggests that the SSN6 protein is functionally related to the SNF1 protein kinase. One possible model is that the SSN6 protein is a target of this protein kinase. We therefore tested whether the SSN6 protein is phosphorylated in vivo. Wild-type cells were metabolically labeled with $^{32}P_i$, either while growing exponentially in glucose or immediately after a shift to conditions of glucose deprivation. Proteins were immunoprecipitated with affinity-purified anti-SSN6 antibody and analyzed by SDS-polyacrylamide gel electrophoresis. The SSN6 protein was phosphorylated in vivo in both glucose-repressed and derepressed cells (Fig. 6A, lanes a and b). This experiment does not address the possibility that specific residues are differentially phosphorylated under the two conditions.

To identify the phosphorylated amino acid residues, the labeled SSN6 proteins were recovered from the gel and subjected to phosphoamino acid analysis (10). Phosphoserine was detected (Fig. 6B). It is possible that threonine and tyrosine are phosphorylated to a much lesser extent.

To determine whether the phosphorylation of SSN6 is dependent on the SNF1 protein kinase, the SSN6 protein from *snf1* mutant cells was examined. The protein was phosphorylated under both growth conditions (Fig. 6A, lanes e to h). It was difficult to assess quantitative differences in the level of SSN6 phosphorylation between *snf1* mutant and wild-type strains because the incorporation of labeled P_i differed dramatically; however, the SNF1 kinase did not appear to be responsible for the majority of the phosphorylation events. In another experiment, a different labeling

TABLE 4. Invertase activity in *snf1* mutants carrying different *SSN6* alleles

Relevant genotype[a]	Invertase activity[b]	
	Repressed	Derepressed
SSN6 snf1	<1	<1
ssn6-Δ7 snf1-Δ3	<1	<1
SSN6-4R309 snf1-K84R	3	8
ssn6-4::URA3 snf1-28	330	1,120

[a] *snf1* alleles have been described previously (5, 7, 8); all confer the same phenotypes and prevent derepression of invertase. Strains are congenic.

[b] Micromoles of glucose released per minute per 100 mg (dry weight) of cells; values are the averages of determinations for at least two strains; standard errors, <20%, except 35% for value of 3.

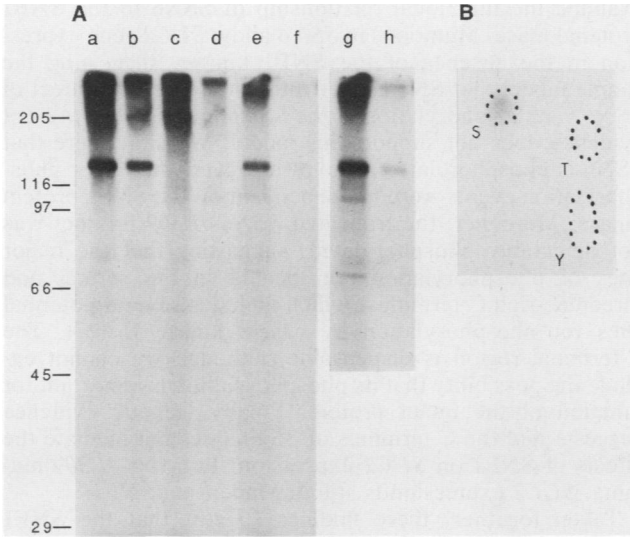

FIG. 6. Phosphorylation of SSN6 protein and phosphoamino acid analysis. (A) Phosphorylation of SSN6 protein. Cultures were metabolically labeled with $^{32}P_i$ during growth in 2% glucose (lanes a, c, and e) or after a shift to 0.15% glucose (lanes b, d, and f). Similar results were obtained with cells grown in 2% raffinose. Proteins were prepared, immunoprecipitated with affinity-purified anti-SSN6 antibody, separated by electrophoresis in SDS-polyacrylamide (10%), and detected by autoradiography for 19 h. Strains used were as follows: lanes a and b, MCY1093 (*SSN6 SNF1*); lanes c and d, MCY1740 (*SSN6-4R309 SNF1*); lanes e and f, MCY1826 (*SSN6 snf1-K84R*); lanes g and h, same as lanes e and f but with longer exposure. Lane a contained threefold more radioactivity than lane b. The amounts of radioactivity loaded in lanes c and d were comparable to those in lanes a and b, respectively. The *snf1* mutant cells labeled much less efficiently, and lanes e and f contained 15- and 100-fold fewer counts per minute than lane a. (B) Phosphoamino acid analysis. The band containing the labeled SSN6 protein was excised from gel lane a. The gel was rehydrated, and protein was eluted (1). The protein was hydrolyzed in 5.7 M HCl at 100°C for 2 h and subjected to thin-layer electrophoresis (10). Positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards are circled. Similar results were obtained for SSN6 protein recovered from lane b (not shown).

regimen resulted in comparable incorporation of $^{32}P_i$ into *snf1* and wild-type strains, and the level of phosphorylation of SSN6 was comparable (unpublished results).

The C terminus of the SSN6 protein is rich in serine and threonine residues that are likely sites for phosphorylation. We therefore examined the phosphorylation of the truncated polypeptide encoded by *SSN6-4R309*. No phosphorylation was detected (Fig. 6A, lanes c and d), even with a 25-fold longer exposure (not shown). In addition, no phosphorylated polypeptide was detected in a strain carrying *SSN6-4R309ΔC* on a high-copy-number plasmid (unpublished results). These findings suggest that the majority, if not all, of the stably phosphorylated residues lie in the C-terminal region.

***SSN6* affects minichromosome maintenance.** Four other members of the TPR family function in mitosis (*CDC16*, *CDC23*, *nuc2*$^+$, and *bimA*), and *CDC16* has been shown to affect the fidelity of mitotic chromosome transmission (16). The *ssn6* mutants are temperature-sensitive for growth at 37°C and grow slowly at 30°C, although no specific mitotic defect has been identified. In addition, *tup1* mutations resemble *ssn6* in their spectrum of pleiotropic phenotypes and genetic behavior (30, 38, 49, 51, 59, 66) and have been shown to affect minichromosome maintenance in *S. cerevisiae* (61). Taken together, this evidence prompted us to examine the effects of *SSN6* on the stability of low-copy-number plasmids containing *CEN* and *ARS* sequences. Isogenic strains carrying different *SSN6* alleles were transformed with plasmids YCp50 (carrying *URA3*, *CEN4*, and *ARS1* [47]), pRS316 (*URA3*, *CEN6*, and *ARSH4* [55]), and pRS315 (*LEU2*, *CEN6*, and *ARSH4* [55]). Transformants were assayed for plasmid stability (32), as described in Materials and Methods. The plasmids were maintained in 45 to 60% of the wild-type cells after 10 to 15 generations of growth under nonselective conditions. In contrast, the plasmids were maintained in only 5 to 20% of the *ssn6-Δ9* mutant cells (Table 5). The values obtained for the mutants represent an upper estimate of the stability because small cell clumps could not be dispersed completely (see Materials and Methods). The *SSN6-4R309ΔC* and *SSN6-Δ11* strains showed intermediate levels of stability.

TABLE 5. Effects of *SSN6* alleles on plasmid maintenance

Experiment	Relevant genotype	Plasmid	Transformant no.	Stability[a] (%)
A	*SSN6*	YCp50	1[b]	50
			2	58
	SSN6	pRS316	1	59
			2	57
	ssn6-Δ9	YCp50	1[b]	6
			1[b]	6
			2[b]	17
			2[b]	9
	ssn6-Δ9	pRS316	1	14
			2	20
B	*SSN6*	YCp50	1[b]	45
	ssn6-Δ9	YCp50	3	17
	SSN6-4R309ΔC	YCp50	1	22
			2	32
	ssn6-Δ9 ura3::pJSΔ11	pRS315	1	38
			2	32

[a] Determined as described in Materials and Methods.
[b] Transformant assayed twice.

DISCUSSION

Mutations in the *SSN6* gene cause a variety of pleiotropic phenotypes, suggesting that the *SSN6* gene product functions as a negative regulator of the expression of a broad spectrum of genes. *SSN6* is required for normal growth of cells at 30°C and is essential for growth at elevated temperatures. Recently, sequence comparison revealed that *SSN6* encodes a member of a family of structurally similar proteins containing the repeated TPR motif (54). Here, we have used specific antisera to characterize the SSN6 protein, and we have used genetic methods to identify the N-terminal region as the important functional domain.

Using anti-SSN6 sera, we identified the *SSN6* gene product as a protein with an apparent molecular mass of 135 kDa. The size predicted from the sequence was 107 kDa. The evidence suggests that C-terminal sequences are responsible for the aberrant migration: the *SSN6-Δ10* product also migrated more slowly than expected (as 110 rather than 85 kDa), whereas the *SSN6-4R309*, *SSN6-4R402*, and *SSN6-lacZ* products all migrated as expected.

Immunofluorescence studies showed that the SSN6 protein is localized exclusively in the nucleus. This finding

suggests that SSN6 acts by a fairly direct mechanism to repress gene expression. We have been unable to demonstrate any DNA-binding activity for this protein, despite extensive efforts (unpublished results). We therefore presently favor models in which SSN6 associates with or affects the activity of DNA-binding proteins. At least two other proteins containing TPR repeats, the *nuc2*$^+$ and *SKI3* products, are also nuclear, and the *nuc2*$^+$ product copurifies with a nuclear scaffold-like fraction (19, 45).

Genetic evidence indicates that the N-terminal third of the SSN6 protein containing the TPR motifs is the region that is important for function. The *SSN6-4R309* product is truncated midway through TPR unit 8 and yet apparently provides wild-type SSN6 function. The conclusion that the N-terminal region is the functional domain is supported by analyses of three other nonsense (*SSN6-4Rn*) alleles, *ssn6-Δ7*, and two *SSN6-lacZ* fusions. Moreover, a deletion removing most of the TPR units (*ssn6-ΔTPR*) caused a mutant phenotype. Three or four TPR units proved sufficient in the *SSN6-Δ11* product. It may be significant that this allele retains the TPR unit containing a conserved Gly residue that is essential in *nuc2*$^+$ (20).

Although the C-terminal two-thirds of the protein is dispensable for the major functions of SSN6, a functional role cannot formally be excluded since this region may affect phenotypes not assayed here. It is possible that the C terminus affects interactions of SSN6 with other proteins or affects the stability of the protein. The C terminus (codons 695 to 966) has a high content of PEST residues (46), comprising 8% proline, 25% serine and threonine, and 18% glutamate and aspartate, and includes five long stretches of PEST residues flanked by basic residues. These features are characteristic of many proteins that have short half-lives (46). It is also noteworthy that the C-terminal 120 residues are highly charged (38%) and acidic overall (net charge, −21). Phosphorylation of the C-terminal region would further increase the charge density.

A striking feature of the SSN6 protein is the presence of long homogeneous tracts of polyglutamine and poly(glutamine-alanine), which have also been found in a variety of other regulatory proteins. These tracts appear to be dispensable even when the protein is not truncated: the *SSN6-Δ10* allele provides wild-type SSN6 function with respect to the phenotypes assayed here. Analyses of the *SSN6-Δ8*, *SSN6-4Rn*, and *SSN6(407)-lacZ* alleles also indicate that the tracts in the middle of the protein are dispensable. However, the *SSN6-Δ10* product, although lacking all polyamino acid tracts, still retains a short region (codons 596 to 682) rich in glutamine (33%) and proline (14%). This region includes seven repeats of the sequence Pro-X-X-X-Gln, where X is uncharged. Glutamine- and proline-rich sequences are common in transcription factors and have been shown to mediate transcriptional activation (11, 34).

This study provides numerous examples of mutant alleles and gene fusions in which the phenotype depends not only on the *SSN6* sequences included, but also on the particular novel junction, fusion, or truncation. For example, the *ssn6-4::URA3* allele contains sequences sufficient for SSN6 function but encodes a nonfunctional product. The polypeptide terminates with 13 residues encoded by the *URA3* insertion, of which 8 are phenylalanine or tyrosine; perhaps this unusual C terminus affects protein folding or function. Thus, examining a variety of mutant constructs proved useful in assessing the functional significance of sequences in this protein.

We have carried out biochemical and genetic studies to examine the functional relationship of SSN6 to the SNF1 protein kinase. Mutations in *SSN6* allow *SUC2* gene expression in the absence of the SNF1 kinase, suggesting the simple model that SNF1 inactivates the repressive effect of SSN6 via phosphorylation of the SSN6 protein. The present evidence does not support this model. We show here that SSN6 is phosphorylated in vivo; however, the major phosphorylation events were not dependent on the SNF1 protein kinase. Moreover, the truncated *SSN6-4R309* product was not detectably phosphorylated, suggesting that the major sites of phosphorylation are located in the serine- and threonine-rich C terminus, which includes several potential sites for phosphorylation by casein kinase II (27). The C-terminal region is dispensable, although we cannot exclude the possibility that its phosphorylation is important for function of the intact protein. Finally, genetic evidence suggests that the C terminus of SSN6 does not mediate the effects of SNF1 on *SUC2* expression. In *SSN6-4R309* mutants, *SUC2* expression is still dependent on *SNF1*.

Taken together, these findings suggest that the SNF1 protein kinase does not affect *SUC2* expression via phosphorylation of the SSN6 protein. This conclusion is subject to the reservation that low-level phosphorylation of the *SSN6-4R309* product may have gone undetected in our experiments. We currently favor models in which the SNF1 protein kinase phosphorylates an as yet unidentified protein that inactivates or antagonizes the repressive action of the SSN6 protein. Such models easily accommodate the genetic evidence that *ssn6* mutations affect expression of genes that are not affected by *snf1* and that *ssn6* mutations do not suppress all of the defects caused by *snf1* (6, 52).

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LITERATURE CITED

1. **Beemon, K., and T. Hunter.** 1978. Characterization of Rous sarcoma virus *src* gene products synthesized in vitro. J. Virol. **28**:551–566.
2. **Bhown, A. S., and J. C. Bennett.** 1983. High-sensitivity sequence analysis of proteins recovered from sodium dodecyl sulfate gels. Methods Enzymol. **91**:450–455.
3. **Boeke, J. D., F. LaCroute, and G. R. Fink.** 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. **197**:345–346.
4. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8**:17–24.
5. **Carlson, M., B. C. Osmond, and D. Botstein.** 1981. Mutants of yeast defective in sucrose utilization. Genetics **98**:25–40.
6. **Carlson, M., B. C. Osmond, L. Neigeborn, and D. Botstein.** 1984. A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107**:19–32.
7. **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.
8. **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.
9. **Celenza, J. L., L. Marshall-Carlson, and M. Carlson.** 1988. The yeast *SNF3* gene encodes a glucose transporter homologous to the mammalian protein. Proc. Natl. Acad. Sci. USA **85**:2130–2134.
10. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99**:387–402.
11. **Courey, A. J., and R. Tjian.** 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55**:887–898.
12. **Fassler, J. S., and F. Winston.** 1989. The *Saccharomyces cerevisiae SPT13/GAL11* gene has both positive and negative regulatory roles in transcription. Mol. Cell. Biol. **9**:5602–5609.
13. **Fjose, A., W. J. McGinnis, and W. J. Gehring.** 1985. Isolation of a homoeo box-containing gene from the *engrailed* region of *Drosophila* and the spatial distribution of its transcripts. Nature (London) **313**:284–289.
14. **Goldstein, A., and J. O. Lampen.** 1975. β-D-Fructofuranoside fructohydrolase from yeast. Methods Enzymol. **42**:504–511.
15. **Harlow, E., and D. Lane.** 1988. Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Hartwell, L. H., and D. Smith.** 1985. Altered fidelity of mitotic chromosome transmission in cell cycle mutants of *S. cerevisiae*. Genetics **110**:381–395.
17. **Heuterspreute, M., J. Orberto, V. H. Thi, and J. Davison.** 1985. Vectors with restriction-site banks. III. *Escherichia coli-Saccharomyces cerevisiae* shuttle vectors. Gene **34**:363–366.
18. **Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2**:163–167.
19. **Hirano, T., Y. Hiraoka, and M. Yanagida.** 1988. A temperature-sensitive mutation of the *Schizosaccharomyces pombe* gene *nuc2*$^+$ that encodes a nuclear scaffold-like protein blocks spindle elongation in mitotic anaphase. J. Cell Biol. **106**:1171–1183.
20. **Hirano, T., N. Kinoshita, K. Morikawa, and M. Yanagida.** 1990. Snap helix with knob and hole: essential repeats in S. pombe nuclear protein *nuc2*$^+$. Cell **60**:319–328.
21. **Hoffman, C. S., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57**:267–272.
22. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
23. **Julius, D., L. Blair, A. Brake, G. Sprague, and J. Thorner.** 1983. Yeast α factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. Cell **32**:839–852.
24. **Julius, D., A. B. MacDermott, R. Axel, and T. M. Jessell.** 1988. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. Science **241**:558–564.
25. **Julius, D., R. Schekman, and J. Thorner.** 1984. Glycosylation and processing of prepro-α-factor through the yeast secretory pathway. Cell **36**:309–318.
26. **Kleid, D. G., D. Yansura, B. Small, D. Dowbenko, D. M. Moore, M. J. Grubman, P. D. McKercher, D. O. Morgan, B. H. Robertson, and H. L. Bachrach.** 1981. Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. Science **214**:1125–1129.
27. **Kuenzel, E. A., J. A. Mulligan, J. Sommercorn, and E. G. Krebs.** 1987. Substrate specificity determinants for casein kinase II as deduced from studies with synthetic peptides. J. Biol. Chem. **262**:9136–9140.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
29. **Laughon, A., A. M. Boulet, J. R. Bermingham, Jr., R. A. Laymon, and M. P. Scott.** 1986. Structure of transcripts from the homeotic Antennapedia gene of *Drosophila melanogaster*: two promoters control the major protein-coding region. Mol. Cell. Biol. **6**:4676–4689.
30. **Lemontt, J. F., D. R. Fugit, and V. L. MacKay.** 1980. Pleiotropic mutations at the *TUP1* locus that affect the expression of mating-type-dependent functions in *Saccharomyces cerevisiae*. Genetics **94**:899–920.
31. **Lillie, S. H., and S. S. Brown.** 1987. Artifactual immunofluorescent labelling in yeast, demonstrated by affinity purification of antibody. Yeast **3**:63–70.
32. **Maine, G. T., P. Sinha, and B.-K. Tye.** 1984. Mutants of *Saccharomyces cerevisiae* defective in the maintenance of minichromosomes. Genetics **106**:365–385.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Mermod, N., E. A. O'Neill, T. J. Kelly, and R. Tjian.** 1989. The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. Cell **58**:741–753.
35. **Miesfeld, R., S. Rusconi, P. J. Godowski, B. A. Maler, S. Okret, A.-C. Wikstrom, J.-A. Gustafsson, and K. R. Yamamoto.** 1986. Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell **46**:389–399.
36. **Morris, N. R.** 1976. Mitotic mutants of *Aspergillus nidulans*. Genet. Res. **26**:237–254.
37. **Myers, A. M., A. Tzagoloff, D. M. Kinney, and C. J. Lusty.** 1986. Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of *lacZ* fusions. Gene **45**:299–310.
38. **Neigeborn, L., and M. Carlson.** 1987. Mutations causing constitutive invertase synthesis in yeast: genetic interactions with *snf* mutations. Genetics **115**:247–253.
39. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–110.
40. **Orr-Weaver, T. L., J. W. Szostak, and R. J. Rothstein.** 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA **78**:6354–6358.
41. **Pinkham, J. L., J. T. Olesen, and L. P. Guarente.** 1987. Sequence and nuclear localization of the *Saccharomyces cerevisiae* HAP2 protein, a transcriptional activator. Mol. Cell. Biol. **7**:578–585.
42. **Pirrotta, V., E. Manet, E. Hardon, S. E. Bickel, and M. Benson.** 1987. Structure and sequence of the *Drosophila zeste* gene. EMBO J. **6**:791–799.
43. **Poole, S. J., L. M. Kauvar, B. Drees, and T. Kornberg.** 1985. The *engrailed* locus of Drosophila: structural analysis of an embryonic transcript. Cell **40**:37–43.
44. **Pringle, J. R., and L. H. Hartwell.** 1981. The *Saccharomyces cerevisiae* cell cycle, p. 97–142. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Rhee, S.-K., R. Icho, and R. B. Wickner.** 1989. Structure and nuclear localization signal of the SKI3 antiviral protein of *Saccharomyces cerevisiae*. Yeast **5**:149–158.
46. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234**:364–368.
47. **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.
48. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–210.
49. **Rothstein, R. J., and F. Sherman.** 1980. Genes affecting the expression of cytochrome *c* in yeast: genetic mapping and genetic interactions. Genetics **94**:871–889.
50. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
51. **Schamhart, D. H. J., A. M. A. Ten Berge, and K. W. Van De Poll.** 1975. Isolation of a catabolite repression mutant of yeast as a revertant of a strain that is maltose negative in the respiratory-deficient state. J. Bacteriol. **121**:747–752.
52. **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.
53. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1978. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. **Sikorski, R. S., M. S. Boguski, M. Goebl, and P. Hieter.** 1990. A repeating amino acid motif in *CDC23* defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell **60**:307–317.
55. **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.
56. **Silver, P. A., and M. N. Hall.** 1988. Transport of proteins into the nucleus, p. 749–769. *In* R. C. Das and P. W. Robbins (ed.), Protein transfer and organelle biogenesis. Academic Press, Inc., San Diego, Calif.
57. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
58. **Spindler, K. R., D. S. E. Rosser, and A. J. Berk.** 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. J. Virol. **49**:132–141.
59. **Stark, H. C., D. Fugit, and D. B. Mowshowitz.** 1980. Pleiotropic properties of a yeast mutant insensitive to catabolite repression. Genetics **94**:921–928.
60. **Suzuki, Y., Y. Nogi, A. Abe, and T. Fukasawa.** 1988. GAL11 protein, an auxiliary transcription activator for genes encoding galactose-metabolizing enzymes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4991–4999.
61. **Thrash-Bingham, C., and W. L. Fangman.** 1989. A yeast mutation that stabilizes a plasmid bearing a mutated *ARS1* element. Mol. Cell. Biol. **9**:809–816.
62. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
63. **Trumbly, R. J.** 1986. Isolation of *Saccharomyces cerevisiae* mutants constitutive for invertase synthesis. J. Bacteriol. **166**: 1123–1127.
64. **Trumbly, R. J.** 1988. Cloning and characterization of the *CYC8* gene mediating glucose repression in yeast. Gene **73**:97–111.
65. **Wharton, K. A., B. Yedvobnick, V. G. Finnerty, and S. Artavanis-Tsakonas.** 1985. *opa*: a novel family of transcribed repeats shared by the *Notch* locus and other developmentally regulated loci in D. melanogaster. Cell **40**:55–62.
66. **Wickner, R. B.** 1974. Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine-5′-monophosphate into deoxyribonucleic acid in vivo. J. Bacteriol. **117**:252–260.
67. **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.