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Snf1 Protein Kinase Regulates Phosphorylation of the Mig1 Repressor in *Saccharomyces cerevisiae*

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In glucose-grown cells, the Mig1 DNA-binding protein recruits the Ssn6-Tup1 corepressor to glucose-repressed promoters in the yeast *Saccharomyces cerevisiae*. Previous work showed that Mig1 is differentially phosphorylated in response to glucose. Here we examine the role of Mig1 in regulating repression and the role of the Snf1 protein kinase in regulating Mig1 function. Immunoblot analysis of Mig1 protein from a *snf1* mutant showed that Snf1 is required for the phosphorylation of Mig1; moreover, *hxk2* and *reg1* mutations, which relieve glucose inhibition of Snf1, correspondingly affect phosphorylation of Mig1. We show that Snf1 and Mig1 interact in the two-hybrid system and also coimmunoprecipitate from cell extracts, indicating that the two proteins interact in vivo. In immune complex assays of Snf1, coprecipitating Mig1 is phosphorylated in a Snf1-dependent reaction. Mutation of four putative Snf1 recognition sites in Mig1 eliminated most of the differential phosphorylation of Mig1 in response to glucose in vivo and improved the two-hybrid interaction with Snf1. These studies, together with previous genetic findings, indicate that the Snf1 protein kinase regulates phosphorylation of Mig1 in response to glucose.

In *Saccharomyces cerevisiae* the Ssn6 (Cyc8)-Tup1 complex represses transcription of genes regulated by glucose, cell type, oxygen, DNA damage, and other signals (27, 34, 45, 46, 48, 52, 54, 56, 57, 61). Ssn6-Tup1 is recruited to these promoters by specific DNA-binding proteins, including α2-Mcm1, **a**1-α2, Mig1, Mig2, Rox1, and Rgt1 (1, 27, 31, 39, 47, 51), and mediates repression by interacting with chromatin (43) and/or the general transcriptional machinery (20, 21, 41). In this work we have focused on the role of Mig1 in regulating repression by Ssn6-Tup1 in response to the glucose signal.

Mig1 is a Cys_2-His_2 zinc finger protein (36) that binds to the promoters of *SUC*, *GAL*, *MAL*, and other glucose-repressible genes; mutation of Mig1 or its binding sites partially relieves glucose repression (15, 17, 22, 25, 35, 36, 44, 53, 55). A LexA-Mig1 fusion protein represses transcription of a *CYC1-lacZ* reporter containing *lexA* operators. Such repression requires Ssn6-Tup1 and occurs only in glucose-grown cells (47, 51). Mig1 is differentially phosphorylated in response to glucose availability (11, 47), and the localization of Mig1 to the nucleus requires glucose (11). In contrast, no difference in modification was detected for Ssn6 or Tup1 (46, 57), and Ssn6 resides in the nucleus regardless of glucose availability (46). These findings strongly suggest that the recruitment of Ssn6-Tup1 to a promoter by Mig1 is regulated by glucose. However, it remains possible that other mechanisms also contribute to regulation of repression by the Mig1-Ssn6-Tup1 complex. Here we present evidence that LexA-Mig1 confers glucose-regulated repression to a promoter that is not otherwise glucose repressed, thereby excluding any requirement for other promoter-bound glucose-regulated factors. We also show that repression by LexA-Ssn6 is not glucose regulated, indicating that the repressor function of the Ssn6-Tup1 complex is not directly regulated by the glucose signal.

The differential phosphorylation of Mig1 in response to glucose suggests that phosphorylation controls its activity in repression, and genetic evidence implicates the Snf1 (Cat1) protein kinase in regulating Mig1. The Snf1 kinase is activated by glucose starvation and is required for expression of glucose-repressed genes (9, 23, 58, 59). Mig1 is thought to function downstream from Snf1 in the pathway, because a *mig1* mutation suppresses the *snf1* mutant defects in *SUC2* and *GAL1* expression (25, 53). Thus, Snf1 appears to inhibit repression by Mig1. Snf1 also inhibits the function of a hybrid Mig1-VP16 activator in the absence of glucose (37). Deletion analysis of Mig1 defined regions that both inhibit repression by Mig1 in the absence of glucose and confer inhibition of Mig1-VP16 by Snf1 (37). Finally, mutation of *SNF1* causes constitutive nuclear localization of Mig1 (11).

In this study, we have examined the role of the Snf1 protein kinase in regulating Mig1 function. We show that Snf1 is required for the phosphorylation of Mig1 in vivo and that the two proteins interact in the two-hybrid system and coimmunoprecipitate. We present evidence that Mig1 is phosphorylated in vitro in a Snf1-dependent reaction. Finally, we show that mutation of four putative Snf1 recognition sites in Mig1 eliminates most of the differential phosphorylation of Mig1 in response to glucose and improves the two-hybrid interaction with Snf1.

MATERIALS AND METHODS

Strains and genetic methods. The *S. cerevisiae* strains used are listed in Table 1. The *Escherichia coli* strains used for propagation of plasmid DNA were XL1-Blue and DH5α. Standard genetic methods were used, and yeast cultures were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids (42).

Oligonucleotides. Oligonucleotides used for PCR are as follows, with serine-to-alanine conversions underlined: OL-H1, 5′-ACTACCATAGCCATGGGCG GCCGCCAAAGCCCATATCCAATG-3′; OL-L1, 5′-TCGAGTGCTGTATAT AAAACCAGTGGTTATATGTACAGTACC-3′; OL-L2, 5′-TCGAGGTACTG TACATATAACCACTGGTTTTATATACAGCAC-3′; OL-S1, 5′-AATAGCC ATAGTGGCGCTAGACTGAAACTGAAC-3′; OL-S2, 5′-ATATTACCAGGT CCGCGAGCTTTAACGGATTTTCAA-3′; OL-S3, 5′-CAGTTGAAGAGACC AGCTGCTGTTTTAAGTTTGAAC-3′; and OL-S4, 5′-ATGCTAAGTAGAG CTGCTGCTGGTACGAATTTGCAC-3′.

Plasmids. To construct pHA-Mig1, the *Sma*I-*Kpn*I fragment from pMIG1 (36) was cloned into the cognate sites of pKB174, a derivative of pRS426 lacking the *Not*I site (2). The resulting plasmid was subjected to site-directed mutagenesis with OL-H1 to introduce a *Not*I site 3′ to the initiating ATG of Mig1. The

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype[a]
MCY829	*MAT*α *ura3 his3 lys2*
MCY2692	*MAT*α *snf1-K84R ura3 his3 leu2*
MCY3278	*MAT*α *reg1*Δ*::URA3 ura3 his3 leu2q*Δ*1 trp1q*Δ*63*
MCY3541	*MAT*α *hxk2*Δ*::URA3 his3 lys2*
MCY3573	*MAT***a** *snf1-K84R mig1q*Δ*2::LEU2 ura3 his3*
MCY3640	*MAT***a** *mig1q*Δ*2::LEU2 ura3 his3 leu2*
MCY3912	*MAT***a** *ura3 his3 lys2 leu2 ade2 trp1*
FY250[b]	*MAT*α *ura3 his3 leu2q*Δ*1 trp1q*Δ*63*
YM4738[c]	*MAT***a** *mig1q*Δ*::ura3::LYS2 mig2q*Δ*1::HIS3 ura3 his3 lys2 ade2 trp1-901 met? can? gal80 LEU2::GAL1-lacZ gal4::GAL4-CAT-TRP1*
CTY10-5d[d]	*MAT***a** *gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1-901*

[a] All MCY strains have the S288C genetic background and carry *SUC2*. Alleles are *ura3-52*, *his3-*Δ*200*, *lys2-801*, *ade2-101*, *leu2-3,112*, and *trp1-*Δ*1* except where noted.
[b] Obtained from F. Winston; S288C genetic background.
[c] Obtained from M. Johnston; S288C genetic background.
[d] Gift of R. Sternglanz (SUNY, Stonybrook, N.Y.).

resulting DNA was digested with *Not*I and ligated to a *Not*I fragment from pGTEP encoding a triple-hemagglutinin (HA) epitope tag (50). pHA-Mig1 partially complements a *mig1* mutation.

To mutate sites in Mig1, the *Bam*HI-*Sal*I fragment from pLexA-Mig1, a derivative of pSH2-1 (47), was cloned into pKB174. Site-directed mutagenesis was carried out by using oligonucleotides OL-S1, -S2, -S3, and -S4. In multiply mutated constructs, alterations were added sequentially and confirmed by restriction digestion or sequence analysis. To make LexA fusions, the *Bam*HI-*Sal*I fragment was then recloned into pSH2-1 (19) or pJH106 (pSH2-1 with *URA3* replacing *HIS3*). pLexA-Mig1ΔZ is pLexA-Mig1 with a deletion between the *Eco*RI site in the polylinker and the *Xho*I site at codon 96 (Fig. 1A). pGAD-Mig1 contains the *Bam*HI-*Sal*I fragment of pLexA-Mig1 cloned into the same sites of pGAD-Not (29). An *Eco*RI-*Sal*I fragment from pGAD-Mig1 was cloned into pACTII (28) (*Eco*RI at codon 88), to create pGAD-Mig1ΔZ. To construct pGAD-Mig1ΔZS222*S278*S311* and pGAD-Mig1ΔZS278*S311*S381*, an *Eco*RI-*Sal*I fragment from the corresponding mutant derivative of pLexA-Mig1 was cloned into pACTII. HA-Snf1 and HA-Snf1K84R were expressed from pSK119 and pSK120, which contain the wild-type and K84R mutant *SNF1* *Bam*HI fragments from pRJ55 and pRJ215, respectively, cloned into pWS93, which expresses a triple-HA epitope from the *ADH1* promoter (a gift of W. Song, Columbia University). pSK117 is derived from pSK37, which is pACTII with the Gal4 activation domain (GAD) deleted, and expresses untagged Snf1. Other proteins were expressed from the following plasmids: $LexA_{87}$, pSH2-1 (19); LexA-Mig1, pLexA-Mig1 (47); GAD, pACTII (28); GAD-Ssn6, pGAD-Ssn6 (47); LexA-Ssn6, CK23 (27); LexA-Snf1, pRJ55 (23); LexA-Snf1K84R, pRJ215 (a gift of R. Jiang, Columbia University); and HA_3, pWS93.

pMT27 contains one LexA operator 5′ to the *HIS3* upstream activation sequence (UAS) in pBM2762 (40). *Sal*I-digested pBM2762 was ligated to a fragment composed of two complementary oligonucleotides (OL-L1 and OL-L2) which recreate a high-affinity ColE1 LexA binding site (12, 26) flanked by mutant *Sal*I sites.

All LexA fusions contain the LexA DNA-binding domain, $LexA_{87}$, except LexA-Snf1 fusions, which contain the entire LexA sequence.

Invertase and β-galactosidase assays. Invertase activity was assayed as previously described (7, 16) and expressed as micromoles of glucose released per minute per 100 mg of cells (dry weight). β-Galactosidase activity was assayed in permeabilized cells (42) and expressed in Miller units (32) or was assayed in protein extracts (8) and expressed as units per milligram of protein (3).

Immunoblot analysis. Cells were grown to mid-log phase in selective SC medium containing 5% glucose (repressed) and derepressed by a shift to 0.05% glucose for 1 h. Cells were collected by centrifugation for 2 min and frozen immediately at −70°C without washing. For Fig. 2C, cells were collected by rapid filtration onto a 0.8-μm-pore-size filter (Micron Separations), and the cell cake was scraped off into methanol at −80°C. Protein extracts were prepared as described previously (8). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting. Primary antibodies were polyclonal LexA antibody (a gift of J. Kamens and R. Brent, Massachusetts General Hospital, Boston) or monoclonal HA antibody (Boehringer Mannheim Biochemical). Antibodies were detected by enhanced chemiluminescence with ECL or ECL Plus reagents (Amersham).

Coimmunoprecipitation assays. Preparation of protein extracts and immunoprecipitation procedures were essentially as described previously (8). The extraction buffer was 50 mM HEPES (pH 7.5)–150 mM NaCl–0.1% Triton X-100–1 mM dithiothreitol–10% glycerol, containing 1 or 2 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Boehringer Mannheim). rProtein A immobilized on Sepharose beads (RepliGen) was added to protein lysates, which were rotated for 20 min and then cleared by centrifugation at 12,000 rpm for 10 min. Anti-HA antibody was added, and samples were mixed for 30 min and cleared by centrifugation for 5 min at 10,000 rpm. The supernatant was mixed with immobilized rProtein A for 1.5 h. The beads were collected by brief centrifugation and washed four times with 1 ml of extraction buffer without protease inhibitor cocktail. The entire procedure was done at 4°C or on ice.

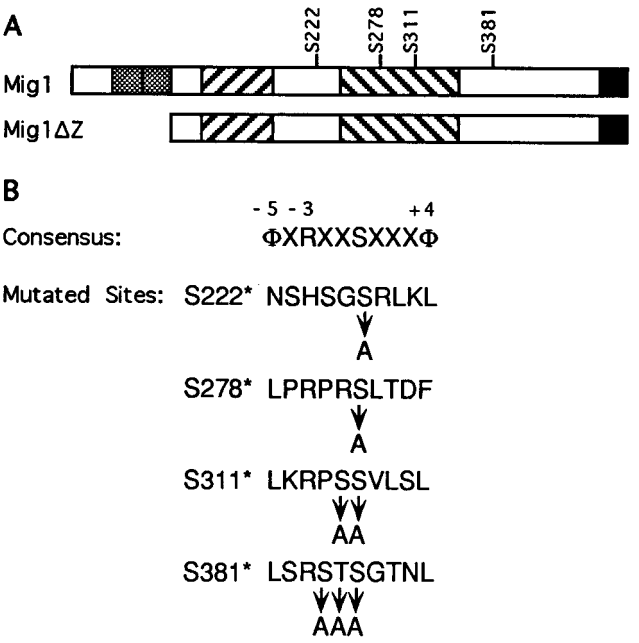

FIG. 1. (A) Structures of Mig1 and Mig1ΔZ. Stippled boxes represent the two zinc fingers. The two regions that are involved in the inhibition of Mig1 function are shown as hatched boxes, and the solid box represents the C-terminal 24 amino acids required for repression (37). Serine residues that are potentially phosphorylated by Snf1 are marked. (B) Potential Snf1 recognition sites. The consensus Snf1 recognition sequence (10) is shown. Φ, hydrophobic residue. Preferred residues: position −5, L > F = I = M > V; position +4, L > I > F > M > V. Potential Snf1 recognition sites in Mig1 are shown and are numbered according to the position of the phosphorylatable serine. The alanine substitutions resulting from site-directed mutagenesis are indicated, and the mutated sites are designated by an asterisk.

Immune complex kinase assays. Preparation of protein extracts and immunoprecipitation were as described above. Beads were then washed in kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Triton X-100) and resuspended in 20 μl of kinase buffer. The kinase reaction was initiated by the addition of 20 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; NEN). Reaction mixtures were incubated at room temperature for 30 min, and reactions were terminated by the addition of 30 μl of 2× sample buffer. Proteins were separated by SDS-PAGE. After electrophoresis, the gel was stained, washed extensively in destaining solution containing 10 mM sodium pyrophosphate, dried, and exposed to film at −70°C with an intensifying screen.

RESULTS

Glucose-regulated repression by LexA-Mig1. Previous work showed that LexA-Mig1 represses transcription of a *lexAop-CYC1-lacZ* reporter only in glucose-grown cells and that repression depends on Ssn6-Tup1 (47, 51) (Table 2). These findings suggested that recruitment of Ssn6-Tup1 by Mig1 is regulated by the glucose signal. However, the *CYC1* promoter responds to glucose, and it remained possible that other factors bound to this reporter contribute to the regulation of repression. To address this issue, we tested the ability of LexA-Mig1 to repress transcription of a reporter driven by the *LEU2* UAS and *HIS3* promoter, with no or one *lexA* operator 5′ to the UAS. In glucose-grown cells, LexA-Mig1 repressed *LEU2-*

TABLE 2. Effects of glucose and *snf1* on repression by LexA fusion proteins

Reporter[a]	Relevant genotype[b]	Expressed protein	Growth condition[c]	β-Galactosidase activity (Miller units)[d]		Fold repression
				Without *lexA* operators	With *lexA* operators	
CYC1-lacZ	WT	LexA-Mig1	High Glu	170	6.8	25
			Raf	1,090	630	1.7
		LexA-Ssn6	High Glu	230	7.6	30
			Raf	620	23	27
		LexA-Mig1ΔZ	High Glu	130	8.1	16
			Low Glu	370	140	2.6
		$LexA_{87}$	High Glu	180	91	1.9
			Low Glu	1,120	390	2.9
			Raf	710	430	1.7
	snf1	LexA-Mig1ΔZ	High Glu	5.1	0.4	13
			Low Glu	4.7	0.3	16
		$LexA_{87}$	High Glu	3.7	1.6	2.3
			Low Glu	2.0	2.0	1.0
LEU2-HIS3-lacZ	WT	LexA-Mig1	High Glu	95	8.3	11
			Raf	72	22	3.3
		$LexA_{87}$	High Glu	94	85	1.2
			Raf	67	21	3.2

[a] The *CYC1-lacZ* reporter contains either no *lexA* operators (pLGΔ312S [18]) or four *lexA* operators (JK1621 [27]) 5′ to the UAS. *LEU2-HIS3-lacZ* contains the *lacZ* gene under the control of the *LEU2* UAS and the *HIS3* promoter with either no *lexA* operators (pBM2762 [40]) or one *lexA* operator (pMT27) 5′ to the UAS.
[b] WT, wild type. Strains were MCY829, MCY3912, and MCY2692.
[c] Strains were grown selectively in 5% glucose (high Glu) or 2% raffinose plus 0.05% glucose (Raf). Cells were also shifted from 5 to 0.05% glucose for 3 h (low Glu).
[d] β-Galactosidase activity was assayed in permeabilized cells. Values represent the averages of results for 3 to 24 transformants. Standard errors were <20%.

HIS3-lacZ expression 11-fold, whereas in raffinose-grown cells, LexA-Mig1 did not repress transcription better than $LexA_{87}$ alone (Table 2); levels of the LexA-Mig1 protein were comparable (data not shown). Thus, glucose-regulated repression by LexA-Mig1 is not promoter specific or dependent on other glucose-regulated factors bound at the reporter.

Mig1 is a likely candidate to mediate the glucose signals controlling repression by Ssn6-Tup1, because Mig1 functions specifically at glucose-regulated promoters, whereas Ssn6-Tup1 serves as a global repressor of differently regulated genes. However, no data exclude the possibility that the repressor function of Ssn6-Tup1 is also regulated by glucose. To test this possibility, we compared the abilities of LexA-Ssn6 (27) to repress transcription in cells grown in glucose or raffinose (Table 2). LexA-Ssn6 repressed *lexAop-CYC1-lacZ* expression 30-fold in glucose-grown cells, consistent with previous results (27). In contrast to LexA-Mig1, LexA-Ssn6 repressed transcription with comparable efficiency (27-fold) in raffinose-grown cells. These findings suggest that regulation of repression is achieved solely via regulated recruitment of Ssn6-Tup1.

Consistent with these findings, we detected a two-hybrid interaction (14) between LexA-Ssn6 and GAD-Mig1ΔZ, a derivative lacking the zinc finger DNA-binding domain (Fig. 1A), in glucose-grown cells but not in raffinose-grown cells (data not shown). The same results were found with LexA-Mig1 or LexA-Mig1ΔZ paired with GAD-Ssn6.

Effect of *snf1* on LexA-Mig1ΔZ function. Genetic evidence suggests that the Snf1 protein kinase inhibits repression by Mig1 during glucose limitation (25, 37, 53). To further examine the role of Snf1 in regulating Mig1 function, we used LexA-Mig1ΔZ, which was stably expressed in a *snf1* mutant; for unknown reasons, LexA-Mig1 was not detectable. In wild-type cells, LexA-Mig1ΔZ conferred glucose-dependent, Ssn6-dependent repression of a reporter (Table 2 and data not shown); thus, the zinc finger domain is dispensable for regulated repression.

LexA-Mig1ΔZ repressed transcription of the *CYC1-lacZ* reporter in glucose-grown *snf1* mutant cells, indicating that the Mig1 repressor function does not require Snf1 (Table 2). To achieve glucose-limiting conditions, we shifted cells from high to low (0.05%) glucose, because a *snf1* mutant does not grow in nonrepressing carbon sources. A shift to low glucose did not relieve repression of *CYC1-lacZ*, consistent with a role for Snf1 in inhibiting repression by Mig1 (Table 2).

Requirement for the Snf1 protein kinase in phosphorylation of Mig1 in vivo. Previously, we showed that LexA-Mig1 is differentially phosphorylated in response to glucose availability (47) (Fig. 2A). To test whether the Snf1 protein kinase is required for this phosphorylation, we used immunoblot analysis to examine tagged Mig1 proteins in a *snf1* mutant. In wild-type cells, LexA-Mig1ΔZ was also differentially modified, similarly to LexA-Mig1 (Fig. 2A). In *snf1* mutant cells, however, the major band migrated at the position predicted for the unmodified protein in both glucose-repressed and derepressed cells (Fig. 2A). We next examined the HA-Mig1 protein expressed from its own promoter. HA-Mig1 was differentially modified in wild-type cells but not in *snf1* mutants (Fig. 2B). In control experiments, a *snf1* mutation did not alter the phosphorylation of an unrelated protein encoded by *SFH1* (4) (data not shown). Thus, the Snf1 protein kinase is required for the phosphorylation of Mig1.

The increased phosphorylation of Mig1 that occurs upon glucose deprivation is compatible with evidence that the Snf1 kinase is more active in glucose-deprived cells (58, 59). However, the phosphorylation observed in glucose-grown cells could result from partial derepression during sample preparation. Because Wilson et al. (58) reported that harvesting of glucose-grown cells by rapid membrane filtration, followed by freezing, minimizes activation of the Snf1 kinase, we also ex-

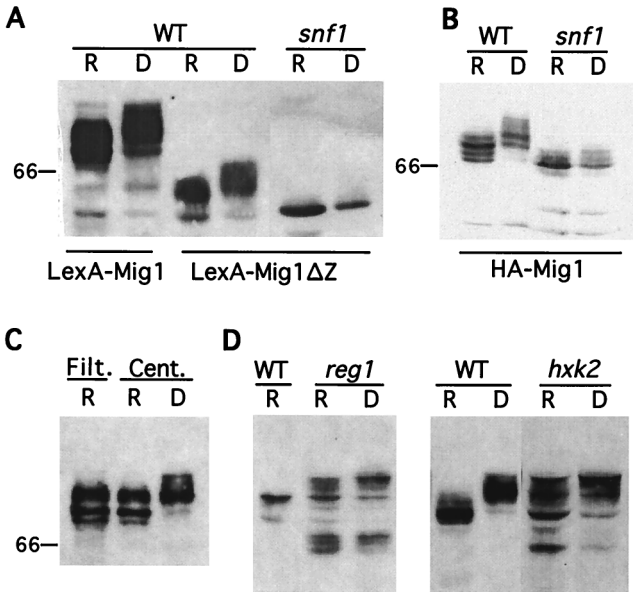

FIG. 2. Immunoblot analysis of Mig1 fusion proteins. Cultures were grown in selective SC medium plus 5% glucose (repressed) (lanes R). Mid-log-phase cultures were derepressed by a shift to SC medium plus 0.05% glucose for 1 h (lanes D). Extracts were prepared, and proteins were separated by SDS-PAGE in 7.5% polyacrylamide and subjected to immunoblot analysis with anti-LexA (A, C, and D) or anti-HA (B). (A and B) Protein extracts (25 and 50 μg for wild-type [WT] and *snf1-K84R* strains, respectively) were prepared from strains MCY829 and MCY2692 transformed by pLexA-Mig1 or pLexA-Mig1ΔZ (A) and pHA-Mig1 (B). (C) Protein extracts (25 μg) were prepared from strain YM4738 expressing LexA-Mig1 from vector pJH106. Cells were collected by rapid membrane filtration (Filt.) or by centrifugation for 2 min (Cent.) (see Materials and Methods). (D) Protein extracts (25 μg for the wild type and 50 μg for *reg1* and *hxk2* strains) were prepared from strains FY250, MCY829, MCY3278, and MCY3541 transformed with pLexA-Mig1. The position of the 66-kDa size marker is indicated.

amined LexA-Mig1 from glucose-grown cells prepared in this manner. The pattern was the same as that obtained with our usual procedure (centrifugation for 2 min without washing, followed by freezing) (Fig. 2C). These findings suggest that the Snf1-dependent phosphorylation observed in glucose-grown cells is unlikely to result from partial derepression, but they cannot exclude this possibility.

In addition, these results strongly suggest that phosphorylation of Mig1 does not occur simply as a consequence of transcriptional activation of an adjacent promoter. LexA-Mig1ΔZ, which lacks the native DNA-binding domain, is still modified in cells with no LexA binding sites.

Phosphorylation of Mig1 in *reg1* and *hxk2* mutants. We next examined mutants in which Snf1 is active in glucose-grown cells. If differential phosphorylation of Mig1 reflects the functional status of Snf1, then mutations that affect the regulation of Snf1 should also influence Mig1 phosphorylation. The *REG1* gene encodes a targeting subunit that directs the function of protein phosphatase 1 in the glucose response (49). Mutation of *REG1* relieves glucose repression of Snf1-dependent genes (24) and causes the Snf1 protein kinase complex to assume an active conformation even in glucose-grown cells (23, 30). Mutation of *HXK2*, encoding hexokinase PII, causes similar phenotypes (23, 24).

Immunoblot analysis showed that the LexA-Mig1 species present in glucose-grown *reg1* and *hxk2* mutants are similar to those found in derepressed cells (Fig. 2D). These results indicate that Reg1 and hexokinase PII affect phosphorylation of Mig1 in a manner consistent with their roles in modulating Snf1 kinase activity.

TABLE 3. Interaction between Snf1 and Mig1 in the two-hybrid system[a]

LexA hybrid	GAD hybrid	β-Galactosidase activity (U/mg of protein)[b]	
		5% Glu	Shift to 0.05% Glu
Snf1	GAD	5	3
Snf1K84R	GAD	4	3
Snf1	Mig1ΔZ	11	72
Snf1K84R	Mig1ΔZ	172	162
Snf1	Mig1ΔZS222*S278*S311*	19	1,110
	Mig1ΔZS278*S311*S381*	11	810
LexA	Mig1ΔZ	9	6
	Mig1ΔZS222*S278*S311*	6	5
	Mig1ΔZS278*S311*S381*	5	7

[a] Strain CTY10-5d was transformed with plasmids expressing the indicated proteins (see Materials and Methods). Immunoblot analysis showed that LexA-Snf1 and LexA-Snf1K84R are expressed at the same levels. GAD-Mig1ΔZ was used because it does not bind to DNA. Transformants were grown to exponential phase in selective SC medium plus 5% glucose (Glu) and then shifted to SC medium plus 0.05% glucose for 3 h.

[b] β-Galactosidase activity was assayed in protein extracts. Values are averages for 3 to 16 transformants, and standard errors were <19%.

Two-hybrid interaction between Mig1 and both wild-type and kinase-dead Snf1 proteins. The preceding data show that Snf1 is required for phosphorylation of Mig1 but do not address whether Snf1 phosphorylates Mig1 directly or controls the phosphorylation of Mig1 by another kinase. To assess the interaction of Snf1 with Mig1 in vivo, we used the two-hybrid system (14). In glucose-grown cells, LexA-Snf1 did not interact significantly with GAD-Mig1ΔZ, but LexA-Snf1K84R interacted strongly (Table 3). The mutant Snf1K84R protein contains a substitution of Arg for the conserved Lys84 in the ATP binding site and exhibits no catalytic activity (8). After cells were shifted to 0.05% glucose for 3 h, β-galactosidase activity could be detected for both combinations. When cells were grown in raffinose, no significant interaction was detected (data not shown), consistent with evidence that Mig1 is cytoplasmic under derepressing conditions (11). These data support the view that Mig1 is a substrate of Snf1 in vivo and suggest that for the wild-type Snf1, glucose deprivation transiently enhances interaction with Mig1.

Coimmunoprecipitation of Snf1 and Mig1. To obtain biochemical evidence for the interaction of Snf1 and Mig1 in vivo, we tested LexA-Snf1 for coimmunoprecipitation with HA-Mig1, expressed from the *MIG1* promoter (Fig. 3). Whole-cell extracts were prepared from cells expressing both proteins, and HA-Mig1 was immunoprecipitated with monoclonal anti-HA antibody. Immunoblot analysis of the precipitate showed that LexA-Snf1 coprecipitated; it was expected that only a small fraction of the LexA-Snf1 would be associated with Mig1. In control experiments, LexA-Snf1 was not detected when the extract contained HA instead of HA-Mig1. Similar results were obtained with LexA-Snf1K84R (Fig. 3); the mutant protein did not coprecipitate better than the wild-type LexA-Snf1, probably because the two-hybrid system and coimmunoprecipitation are not comparable assays.

Snf1-dependent phosphorylation of Mig1 in vitro. We next addressed the ability of Snf1 to phosphorylate Mig1 in vitro. Extracts were prepared from cells expressing HA-Snf1 and LexA-Mig1 from the *ADH1* promoter, and HA-Snf1 was immunoprecipitated with anti-HA. The immune complexes were

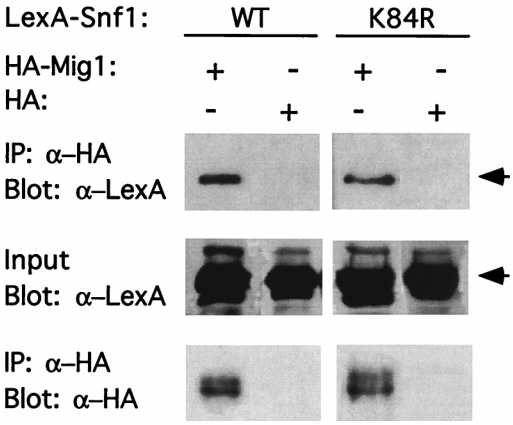

FIG. 3. Coimmunoprecipitation of LexA-Snf1 and LexA-Snf1K84R with HA-Mig1. Strains MCY3573 and MCY3640 were transformed with plasmids expressing LexA-Snf1 and LexA-Snf1K84R, respectively, and either HA-Mig1 (from the *MIG1* promoter) or HA (from the *ADH1* promoter). Protein extracts were prepared from cells grown in glucose, and proteins (200 μg) were immunoprecipitated (IP) with anti (α)-HA antibody, separated by SDS-PAGE in 7.5% polyacrylamide, and immunoblotted with α-LexA (upper panel). The input protein (25 μg) is also shown (middle panel); an arrow indicates the position of the Snf1 fusion. The same immunoblot was reprobed with α-HA to confirm the precipitation of HA-Mig1 (lower panel). No band was detected at the position of LexA-Snf1 when HA-Mig1 was immunoprecipitated from extracts lacking LexA-Snf1. WT, wild type.

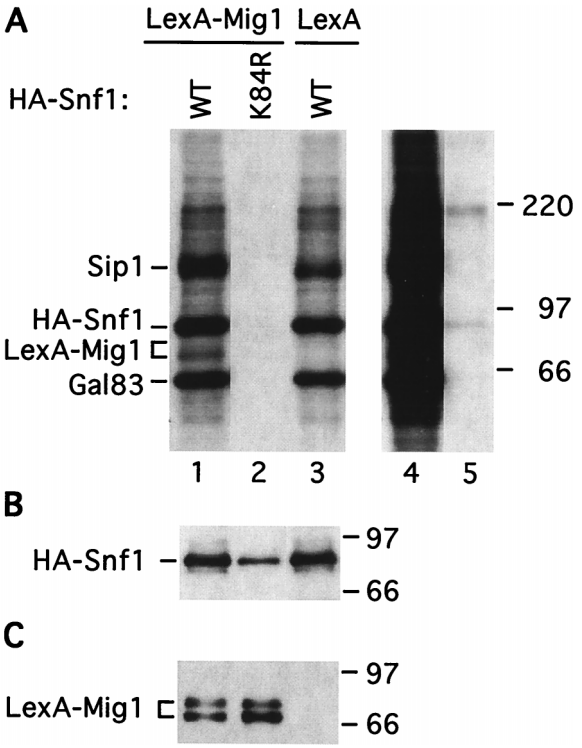

FIG. 4. Snf1-dependent phosphorylation of Mig1 in vitro. Strain FY250 (wild type [WT]) was transformed with plasmids expressing HA-Snf1 or HA-Snf1K84R and LexA-Mig1 or the LexA moiety (from the parental vector). Protein extracts were prepared from cells grown in selective SC medium plus 2% glucose; previous studies have shown that the Snf1 kinase is activated during preparation of the extracts (13, 58). (A and B) Proteins (200 μg) were immunoprecipitated with anti-HA antibody. (A) Half of the immunoprecipitate was resuspended in kinase assay buffer and incubated with [γ-^{32}P]ATP. Proteins were then separated by SDS-PAGE in 8% polyacrylamide, and the gel was subjected to autoradiography to detect phosphorylated products. The left panel (lanes 1 to 3) shows a 3.5-h exposure, and the right panel (lanes 4 and 5) shows a 15-h exposure of lanes 1 and 2. (B) The remaining half of the sample was subjected to SDS-PAGE and immunoblot analysis with anti-HA to confirm the immunoprecipitation of HA-Snf1 and HA-Snf1K84R. We could not reproducibly detect LexA-Mig1 by immunoblot analysis of the immunoprecipitates. (C) Input proteins (10 μg) were analyzed by immunoblot analysis to verify the expression of LexA-Mig1. The phosphorylated LexA-Mig1 product detected in panel A corresponds to the lower band in this panel. Numbers on the right of each panel are molecular masses in kilodaltons.

resuspended in kinase assay buffer and incubated with [γ-^{32}P]ATP. The proteins were separated by gel electrophoresis, and the phosphorylated products were visualized by autoradiography (Fig. 4A). In addition to the products usually detected in such assays, including Snf1, Sip1, and Gal83 (60), a phosphorylated protein corresponding to LexA-Mig1 was detected (Fig. 4A, lane 1). This product was absent in assays of extracts containing only the LexA moiety (expressed from the parental vector) (Fig. 4A, lane 3). Control experiments with the kinase-dead HA-Snf1K84R mutant protein confirmed that the kinase activity detected in this assay was dependent on Snf1 (Fig. 4A, lane 2), and no phosphorylated LexA-Mig1 was detected even upon overexposure (Fig. 4A, lanes 4 and 5). In an independent experiment, we similarly detected phosphorylation of LexA-Mig1 in immune complex assays of the wild-type HA-Snf1, but not the mutant kinase, and also showed that no phosphorylation was detected in controls with untagged Snf1 expressed at the same level (data not shown). Thus, LexA-Mig1 is phosphorylated in vitro in a Snf1-dependent reaction.

Mutation of putative Snf1 phosphorylation sites in Mig1. We identified potential Snf1 phosphorylation sites in Mig1 based on their similarity to the consensus substrate recognition sequence (10), which contains an arginine at position −3 and hydrophobic residues at positions −5 and +4 relative to the phosphorylated serine (Fig. 1B). This consensus sequence was determined by assaying the ability of purified Snf1 kinase to phosphorylate variants of a synthetic peptide that is recognized by the mammalian Snf1 homolog, AMP-activated protein kinase (5, 33).

Three sites in Mig1, containing serine residues S278, S311, and S381, match the consensus site, and the site at S222 resembles the consensus (Fig. 1B). S278 and S311 are located in a regulatory region that appears to mediate the inhibition of Mig1 function by Snf1 (37), and residues 261 to 400 are sufficient to confer glucose-regulated nuclear localization (11). The sites at S222, S278, and S311 are conserved in Mig1 homologs from the yeasts *Kluyveromyces marxianus* and *Kluyveromyces lactis* (6).

We converted the phosphorylatable serine residues to alanine by site-directed mutagenesis and also altered any adjacent serines or threonines (Fig. 1B). The mutant sites are designated with an asterisk. We constructed several combinations of mutant sites including S278* and S311*, because these two sites match the consensus sequence, reside within a regulatory region, and are conserved. The mutant Mig1 proteins were expressed as LexA fusions to facilitate detection of the proteins and assays of repressor function.

Immunoblot analysis indicated that mutation of all four sites eliminated most of the differential phosphorylation of Mig1 in response to glucose (Fig. 5A). Although the triple-mutant proteins still displayed a glucose-dependent shift in mobility, the shift was less pronounced, and the same was true for the S278*S311* double mutant (data not shown). Thus, these sites appear to be phosphorylated in vivo. However, mutation of these sites did not reduce phosphorylation of Mig1 as substantially as did mutation of *SNF1*, suggesting that Mig1 also contains additional sites for Snf1-dependent phosphorylation. We were unable to assess the effect of these mutations on phos-

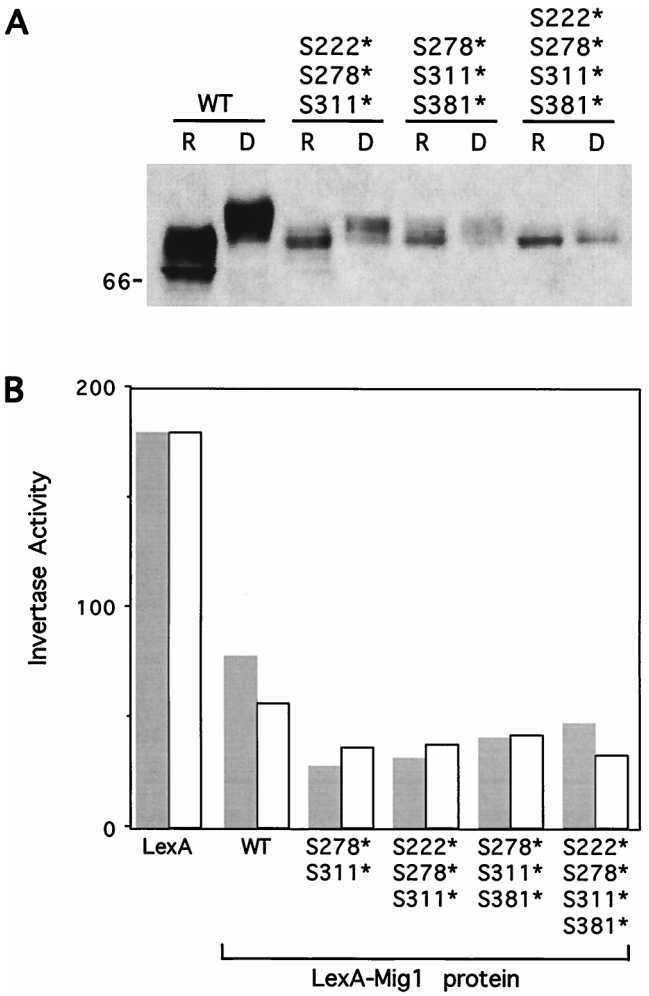

FIG. 5. Analysis of mutant LexA-Mig1 fusion proteins. (A) Strain MCY3640 was transformed with plasmids derived from the vector pJH106. Cultures were grown selectively in SC medium plus 5% glucose (repressed) (lanes R) and were derepressed by a shift to SC medium plus 0.05% glucose for 1 h (lanes D), and protein extracts were prepared. Proteins (25 μg for cells expressing wild-type LexA-Mig1 [WT] and 50 μg for cells expressing mutant LexA-Mig1 proteins) were separated by SDS-PAGE in 7.5% polyacrylamide and subjected to immunoblot analysis with anti-LexA antibody. The position of the 66-kDa size marker is indicated. (B) Transformants of strains MCY3640 (*mig1*Δ) (shaded bars) and YM4738 (*mig1*Δ *mig2*Δ) (open bars) expressing the indicated LexA-Mig1 protein were grown selectively in SC medium plus 2% raffinose and 0.05% glucose. Derepression of *SUC2* was monitored by assaying invertase activity for 4 to 11 transformants. Standard errors were <13%. Immunoblot analysis confirmed that mutant proteins were present at lower levels than wild-type LexA-Mig1 in strain YM4738, consistent with panel A. Similar assays of transformants shifted from 5 to 0.05% glucose for 1 h did not reveal any delay in derepression for strains expressing mutant proteins.

phorylation by Snf1 in immune complex assays due to the low levels of the mutant proteins.

None of the mutated sites is essential for Mig1 repressor function. LexA-Mig1 proteins containing these mutations restored glucose repression of *SUC2* in a *mig1*Δ *mig2*Δ mutant (Table 4); Mig2 is a related zinc finger repressor protein that assists Mig1 (31). The mutant LexA-Mig1 proteins also repressed transcription of the *lexAop-CYC1-lacZ* reporter in glucose-grown cells (Table 4).

We then assayed for release of repression by the mutant LexA-Mig1 proteins in response to glucose limitation. Derepressed invertase activities of *mig1*Δ and *mig1*Δ *mig2*Δ cells

TABLE 4. Repression by mutant LexA-Mig1 proteins

Expressed protein	Invertase activity[a]	β-Galactosidase activity[b] with:		Fold repression
		0 *lexA* operators	4 *lexA* operators	
$LexA_{87}$	83	322	151	2.1
LexA-Mig1	4	244	8	30
LexA-Mig1S278*S311*	6	ND[c]	ND	ND
LexA-Mig1S222*S278*S311*	8	547	25	22
LexA-Mig1S278*S311*S381*	7	431	21	21

[a] YM4738 (*mig1*Δ *mig2*Δ) was transformed with plasmids expressing the indicated LexA fusion protein. The double mutant produces higher invertase activity than a *mig1*Δ single mutant, thereby providing a more sensitive assay for Mig1 repressor function. Transformants were grown to mid-log phase in SC medium plus 5% glucose and assayed for invertase activity (expressed as micromoles of glucose released per minute per 100 mg of cells).

[b] Wild-type strain MCY829 was transformed with plasmids expressing the indicated LexA fusion protein and *lexAop-CYC1-lacZ* reporters with zero or four *lexA* operators (pLGΔ312S, JK1621). Transformants were grown in SC medium plus 5% glucose. β-Galactosidase activity (Miller units) was assayed in permeabilized cells. Values represent the averages from at least three transformants. Standard errors were <18%.

[c] ND, not determined.

expressing the mutant proteins were 1.6- to 2.8-fold and 1.4- to 1.7-fold lower, respectively, than that of cells expressing the wild-type LexA-Mig1 (Fig. 5B). The levels of the mutant LexA-Mig1 proteins were reproducibly lower than that of the wild-type protein (twice as much protein was loaded for the mutant extracts in Fig. 5A; data not shown for *mig1*Δ *mig2*Δ transformants). In view of the decreased abundance of the mutant proteins, the effects of the mutations on release of repression of *SUC2* may be substantial. Protein levels are likely to be important, because overexpression of wild-type LexA-Mig1 reduced derepression relative to the case for the control with LexA.

Finally, we determined the effects of these mutations on the two-hybrid interaction of Mig1 with Snf1. We reasoned that if a mutation abolishing the Snf1 catalytic activity (K84R) improves detection of this interaction, then mutations that prevent phosphorylation of Snf1 recognition sites might also affect interaction. In glucose-grown cells, mutant derivatives of GAD-Mig1ΔZ showed weak interaction with LexA-Snf1; however, substantial β-galactosidase activity was produced after a shift to low glucose, which activates the Snf1 kinase. The two mutant proteins interacted strongly with Snf1, producing 1,110 and 810 U of activity, compared to 72 U for the wild-type GAD-Mig1ΔZ (Table 3). Thus, Ser-to-Ala substitutions in these putative Snf1 recognition sites increased the two-hybrid interaction between Mig1 and Snf1 11- to 15-fold.

DISCUSSION

Previous evidence suggested that Mig1 recruits the Ssn6-Tup1 corepressor to glucose-repressed promoters in response to the glucose signal. Here we have further examined the role of Mig1 in regulating repression. First, we show that LexA-Mig1 confers glucose-regulated repression to a *LEU2-HIS3-lacZ* reporter, thereby excluding any requirement for other regulatory factors specific to glucose-regulated reporters. Similar studies of LexA-Mig1ΔZ further indicate that the zinc finger region is not required for regulated repression. Second, we show that repression by LexA-Ssn6 is not regulated by glucose. These experiments substantiate the model that regulation is achieved by the regulated recruitment of Ssn6-Tup1 by Mig1.

The differential phosphorylation of Mig1 in response to glucose suggested that phosphorylation regulates its repressor function (11, 47), and genetic evidence indicated that the Snf1 protein kinase inhibits Mig1 function during glucose starvation (25, 37, 53). Here we present evidence that Snf1 regulates the phosphorylation of Mig1. We show that modification of Mig1 is dramatically reduced in a *snf1* mutant, indicating that Snf1 is required for the phosphorylation of Mig1. Consistent with these observations, Snf1 kinase activity increases in glucose-limited cells (58, 59). Conversely, in glucose-grown *hxk2* and *reg1* mutants, which are defective in glucose inhibition of the Snf1 kinase activity (23) and glucose repression of Snf1-dependent genes (24), the migration patterns of LexA-Mig1 resemble that of the derepressed wild type. These data strongly suggest that the regulation of Snf1 kinase activity is coupled to the regulation of Mig1 modification, with the caveat that *hxk2* and *reg1* may also affect Mig1 by other mechanisms. During the preparation of this paper, the Snf1-dependent phosphorylation of a Mig1-VP16 protein containing the N-terminal two-thirds of Mig1 (residues 1 to 351) was reported; however, this truncated Mig1 fusion differs from the full-length Mig1 proteins examined here in that it is not phosphorylated in glucose-grown cells (38).

Several lines of genetic and biochemical evidence support the view that Snf1 phosphorylates Mig1 in vivo. First, Snf1 and Mig1 interact in the two-hybrid system. Moreover, the kinase-dead mutant Snf1K84R gives a stronger signal than wild-type Snf1, and mutant Mig1 proteins with Ser-to-Ala substitutions in consensus Snf1 recognition sites give a stronger signal than wild-type Mig1. A shift to low glucose causes an increase in interaction between wild-type Snf1 and Mig1, presumably transient because no interaction was detected in cells grown in raffinose. Second, Snf1 coimmunoprecipitates with Mig1 from cell extracts. Third, mutation of all four putative Snf1 recognition sites eliminates most of the differential phosphorylation of Mig1 in response to glucose. Finally, functional assays of the mutant LexA-Mig1 proteins revealed defects of up to 2.8-fold in release of repression of *SUC2*, and the magnitude is most likely underestimated due to the reduced levels of the mutant proteins. Studies of Mig1-VP16 similarly showed that mutation of serines 278, 310, and 311 affects its phosphorylation and reduces the Snf1 dependence of its activation function 3.8-fold, although protein levels were not reported (38).

These studies of the relationship of Snf1 and Mig1 in vivo are further supported by in vitro evidence that immunoprecipitated Snf1 kinase phosphorylates coprecipitated Lex-Mig1. This reaction was dependent on Snf1 activity, and no phosphorylation was detected in immune complex assays of Snf1K84R. The simple interpretation is that Mig1 is phosphorylated by Snf1, but a more complicated scenario, in which Snf1 is still intimately involved in the phosphorylation of Mig1, cannot be excluded. It is possible that Snf1 phosphorylates and activates an associated Snf1-dependent kinase, which then phosphorylates Mig1; however, this model is difficult to reconcile with the effects of mutations in Mig1 on its phosphorylation and two-hybrid interaction with Snf1.

Although most of the phosphorylation of Mig1 in vivo depends on the Snf1 kinase, Snf1 may not be directly responsible for all of the phosphorylation events. Mutation of all four Snf1 consensus recognition sites did not reduce phosphorylation of Mig1 nearly as substantially as the *snf1* mutation. Mig1 may contain other Snf1 recognition sites, unrelated to the defined consensus, and/or Snf1 may regulate the phosphorylation of Mig1 by another protein kinase. Consistent with this view, analysis of Mig1-VP16 identified a Snf1-dependent phosphorylation site at serine 108, which does not resemble a Snf1 site (38).

Phosphorylation of Mig1 could regulate the recruitment of Ssn6-Tup1 to a promoter by affecting any of several steps: binding of Mig1 to the promoter, interaction of Mig1 with the Ssn6-Tup1 corepressor, or localization of Mig1 to the nucleus. It is unlikely that DNA binding is regulated, because regulated repression was achieved by LexA-Mig1 bound to *lexA* operators. The possibility that phosphorylation disrupts the interaction of Mig1 with Ssn6-Tup1 has not been addressed, but this cannot be the only mechanism, because Snf1 affects activation by Mig1-VP16 (37) and affects localization of Mig1 in an *ssn6* mutant (11). Evidence that the differential localization of Mig1 is Snf1 dependent and correlates with its differential phosphorylation (11) strongly suggests that phosphorylation functions as a regulatory signal for localization.

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