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 Snf6-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, hxxk2 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 Snf6 (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, 59, 61). Snf6-Tup1 is recruited to these promoters by specific DNA-binding proteins, including a2-Mcm1, a1-a2, Mig1, Mig2, Rob1, 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 Snf6-Tup1 in response to the glucose signal.

Mig1 is a Cys2-His2 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 reporter in the two-hybrid system and also coimmunoprecipitates with Snf1 from cell extracts, indicating that the two proteins interact in vivo. Immunoblot analysis 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, Snf1, a serine-threonine protein kinase, is required for glucose repression. Snf1 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' -ACTACCATAGCAGTGCGCG GCCGGCAAGCCCATATCCAG-3'; OL-L1, 5' -TCGAGCTCTGTATGAAAGCAC-3'. OL-L2, 5' -TCGAAGTCTGTTCCAGAAGC-3'. OL-S1, 5' -AAATAGGC ATATGCGCCGCTGACTG GAGTTAACGTGCAG-3'. OL-S2, 5' -ATATACACG GCGCGCTATAGGCTTCTTGGACAC-3'. OL-S3, 5' -ATATACACG GCGCGCTATAGGCTTCTTGGACAC-3'. OL-S4, 5' -ATATACACG GCGCGCTATAGGCTTCTTGGACAC-3'.

Plasmids. To construct pHA-Mig1, the Snf1-KpnI fragment from pMI1G1 (36) was cloned into the cognate sites of pBluescript, a derivative of pBluescript lacking the NorI site (2). The resulting plasmid was subjected to site-directed mutagenesis with OL-H1 to introduce a NorI site 3' to the initiating ATG of Mig1. The
resulting DNA was digested with NotI and ligated to a NotI fragment from pGTEP encoding a triple-hemagglutinin (HA) epitope tag (50). pHA-Mig1 partially complements a mig1 mutation.

To mutate sites in Mig1, the BamHI-SalI fragment from pLexA-Mig1, a derivative of pSH2-3 (47), was cloned into pKB174. Site-directed mutagenesis was carried out by using oligonucleotides OL-S1, -S2, -S3, and -S4. In multiply mutant constructs, alterations were added sequentially and confirmed by restriction digestion or sequence analysis. To make LexA fusions, the BamHI-SalI fragment was then recloned into pSH2-1 (19) or pSH106 (pSH2-1 with URA3 replacing HSP3). pLexA-Mig1AZ is pLexA-Mig1 with a deletion between the EcoRI site in the polylinker and the XhoI site at codon 96 (Fig. 1A). pGAD-Mig1 contains the BamHI-SalI fragment of pLexA-Mig1 cloned into the same sites of pGAD-Not (29). An EcoRI-SalI fragment from pGAD-Mig1 was cloned into pACTII (28) (EcoRI at codon 88), to create pGAD-Mig1AZ. To construct pGAD-Mig1AZS222, S278*S311* and pGAD-Mig1AZS278*S311*S381*, an EcoRI-SalI fragment from the corresponding mutant derivative of pLexA-Mig1 was cloned into pACTII. HA-Snf1 and HA-Snf1K84R were expressed from pSK117 and pSK120, which contain the wild-type and K84R mutant SNF1 BamHI 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 pGACTII with the Gal4 activation domain (GAD) deleted, and expresses untagged Snf1. Other proteins were expressed from the following plasmids: LexA-Ssn6, CK23 (27); LexA-Snf1, pRJ55 (23); LexA-Snf1K84R, pRJ215 (a gift of R. Jiang, Columbia University); and HA-, pWS93. pMT27 contains one LexA operator 5′ to the HIS3 upstream activation sequence (UAS) in pMB2762 (40). SalI-digested pMB2762 was ligated to a fragment resulting from two complementary oligonucleotides (OL-L1 and OL-L2) which recombine a high-affinity CoElI LexA binding site (12, 26) flanked by mutant SalI sites. All LexA fusions contain the LexA DNA-binding domain, LexApol, 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 with washing. For Fig. 2C, cells were collected by rapid filtration onto a 0.8-μm-pore-size filter (Milcon 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 (Amer sham).

**Commmunoprecipitation assays.** Preparation of protein extracts and immunoprecipitation procedures were essentially as described previously (8). The extract 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). Protein 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.

**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 MgCl2, 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-32P]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 Sn6-Tup1 (47, 51) (Table 2). These findings suggested that recruitment of Sn6-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-
D\textsuperscript{T}up1. repression is achieved solely via regulated recruitment of Ssn6-
ous results (27). In contrast to LexA-Mig1, LexA-Ssn6 expression 30-fold in glucose-grown cells, consistent with previ-
expression. To test this possibility, we compared the abilities of LexA-Ssn6 to repress transcription in cells grown in glucose or raf-
To further examine the role of Snf1 in regulating Mig1 function, we used LexA-
Mig1\DeltaZ, which was stably expressed in a \textit{snf1} mutant; for unknown reasons, LexA-Mig1 was not detectable. In wild-type cells, LexA-Mig1\DeltaZ conferred glucose-dependent, Ssn6-de-
dependent repression of a reporter (Table 2 and data not shown);
thus, the zinc finger domain is dispensable for regulated re-
represents the averages of results for 3 to 24 transformants. Standard errors were <20%.

**TABLE 2.** Effects of glucose and \textit{snf1} on repression by LexA fusion proteins

<table>
<thead>
<tr>
<th>Reporter\textsuperscript{a}</th>
<th>Relevant genotype\textsuperscript{b}</th>
<th>Expressed protein</th>
<th>Growth condition\textsuperscript{c}</th>
<th>(\beta)-Galactosidase activity (Miller units)\textsuperscript{d}</th>
<th>Fold repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYC1-\textit{lacZ}</td>
<td>WT</td>
<td>LexA-Mig1</td>
<td>High Glu</td>
<td>170</td>
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<td></td>
<td></td>
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<td>230</td>
<td>7.6</td>
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<tr>
<td></td>
<td></td>
<td>LexA-Mig1\DeltaZ</td>
<td>High Glu</td>
<td>130</td>
<td>8.1</td>
</tr>
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<td></td>
<td></td>
<td>Low Glu</td>
<td>370</td>
<td>140</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LexA\textit{A}\textsubscript{87}</td>
<td>High Glu</td>
<td>180</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>1,120</td>
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<td></td>
<td></td>
<td>Raf</td>
<td>710</td>
<td>430</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>\textit{snf1}</td>
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<td>0.4</td>
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<tr>
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<td></td>
<td>LexA\textit{A}\textsubscript{87}</td>
<td>High Glu</td>
<td>3.7</td>
<td>1.6</td>
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<tr>
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<td></td>
<td>Low Glu</td>
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<td>2.0</td>
<td>1.0</td>
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<tr>
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<td>LexA-Mig1</td>
<td>High Glu</td>
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<td>8.3</td>
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<tr>
<td></td>
<td></td>
<td>LexA\textit{A}\textsubscript{87}</td>
<td>High Glu</td>
<td>94</td>
<td>85</td>
</tr>
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<td></td>
<td></td>
<td>Raf</td>
<td>67</td>
<td>21</td>
<td>3.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The \textit{CYC1-lacZ} reporter contains either no \textit{lexA} operators (pLG\textit{LG}312S [18]) or four \textit{lexA} operators (JK1621 [27]) 5’ to the UAS. \textit{LEU2-HIS3-lacZ} contains the \textit{lacz} gene under the control of the \textit{LEU2} UAS and the \textit{HIS3} promoter with either no \textit{lexA} operators (pBM2762 [40]) or one \textit{lexA} operator (pMT27) 5’ to the UAS.

\textsuperscript{b} WT, wild type. Strains were MCY829, MCY3912, and MCY2692.

\textsuperscript{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).

\textsuperscript{d} \(\beta\)-Galactosidase activity was assayed in permeabilized cells. Values represent the averages of results for 3 to 24 transformants. Standard errors were <20%.

\textsuperscript{f} 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-
studied 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.

**Commmunoprecipitation of Snf1 and Mig1.** To obtain biochemical evidence for the interaction of Snf1 and Mig1 in vivo, we tested LexA-Snf1 for communoprecipitation 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 communoprecipitation 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 analyzed by SDS-PAGE followed by Western blot analysis with anti-HA and anti-Mig1 antibodies (Fig. 4). The migration of Mig1 as a 66-kDa protein suggests that Mig1 is a substrate of Snf1 in vivo and in vitro.

**Phosphorylation of Mig1 in reg1 and hxx2 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 HXX2, encoding hexokinase PII, causes similar phenotypes (23, 24).

Immunoblot analysis showed that theLexAMig1 species present in glucose-grown reg1 and hxx2 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.

**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 and LexA-Snf1K84R were 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.

**Table 3. Interaction between Snf1 and Mig1 in the two-hybrid system**

<table>
<thead>
<tr>
<th>Snf1</th>
<th>GAD hybrid</th>
<th>β-Galactosidase activity (U/mg of protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>5% Glu</td>
</tr>
<tr>
<td>Snf1</td>
<td>GAD</td>
<td>5</td>
</tr>
<tr>
<td>Snf1K84R</td>
<td>GAD</td>
<td>4</td>
</tr>
<tr>
<td>Snf1</td>
<td>Mig1ΔZ</td>
<td>11</td>
</tr>
<tr>
<td>Snf1K84R</td>
<td>Mig1ΔZ</td>
<td>172</td>
</tr>
<tr>
<td>Snf1</td>
<td>Mig1ΔZS222<em>S278</em>S311*</td>
<td>19</td>
</tr>
<tr>
<td>Snf1</td>
<td>Mig1ΔZS278<em>S311</em>S381*</td>
<td>11</td>
</tr>
<tr>
<td>LexA</td>
<td>Mig1ΔZ</td>
<td>9</td>
</tr>
<tr>
<td>LexA</td>
<td>Mig1ΔZS222<em>S278</em>S311*</td>
<td>6</td>
</tr>
<tr>
<td>LexA</td>
<td>Mig1ΔZS278<em>S311</em>S381*</td>
<td>5</td>
</tr>
</tbody>
</table>

* 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.

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

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**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 3 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 for wild-type and 50 μg for reg1 and hxx2 strains) were prepared from strains FY250, MCY829, MCY3278, and MCY3541 transformed with pLexA-Mig1. The position of the 66-kDa size marker is indicated.

![Image](http://example.com/image.png)
resuspended in kinase assay buffer and incubated with [γ-32P]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 sequence (11). The mutant sites are designated with an asterisk. We constructed several combinations of the 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-
Derepression was achieved by the regulated recruitment of Ssn6-Tup1 by LexA-Ssn6 in strain YM4738, consistent with panel A. Similar assays of transformants shifted from 5% glucose to 0.05% glucose for 1 h did not reveal any delay in derepression for strains expressing mutant proteins.

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 (pLG4312, 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%.

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 and Mig2, producing 1,110-fold repression relative to the case for the control with LexA.

<table>
<thead>
<tr>
<th>Expressed protein</th>
<th>Invertase activity</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 lexA operators</td>
</tr>
<tr>
<td></td>
<td>Fold repression</td>
</tr>
<tr>
<td>LexA&lt;sub&gt;47&lt;/sub&gt;</td>
<td>83</td>
</tr>
<tr>
<td>LexA-Mig1</td>
<td>4</td>
</tr>
<tr>
<td>LexA-Mig1S278<em>S311</em></td>
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<tr>
<td>LexA-Mig1S222<em>S278</em>S311*</td>
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</tr>
<tr>
<td>LexA-Mig1S278<em>S311</em>S381*</td>
<td>7</td>
</tr>
</tbody>
</table>

*YM4738 (mig1<sup>Δ</sup>mig2<sup>Δ</sup>) was transformed with plasmids expressing the indicated LexA fusion protein. The double mutant produces higher invertase activity than a mig1<sup>Δ</sup> 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).

**DISCUSSION**

Previous evidence suggested that Mig1 recruits the Snf6-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 Snf6-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 mutation 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 hxx2 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 hxx2 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 mutantLexA-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 Snf6-Tup1 to a promoter by affecting any of several steps: binding of Mig1 to the promoter, interaction of Mig1 with the Snf6-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 Snf6-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 snn6 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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