

Activation of the *Saccharomyces cerevisiae* Heat Shock Transcription Factor Under Glucose Starvation Conditions by Snf1 Protein Kinase*

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Heat shock transcription factor (HSF) is an evolutionarily conserved protein that mediates eukaryotic transcriptional responses to stress. Although the mammalian stress-responsive HSF1 isoform is activated in response to a wide array of seemingly unrelated stresses, including heat shock, pharmacological agents, infection and inflammation, little is known about the precise mechanisms or pathways by which this factor is activated by many stressors. The baker's yeast *Saccharomyces cerevisiae* encodes a single HSF protein that responds to heat stress and glucose starvation and provides a simple model system to investigate how a single HSF is activated by multiple stresses. Although induction of the HSF target gene *CUP1* by glucose starvation is dependent on the Snf1 kinase, HSF-dependent heat shock induction of *CUP1* is Snf1-independent. Approximately 165 *in vivo* targets for HSF have been identified in *S. cerevisiae* using chromatin immunoprecipitation combined with DNA microarrays. Interestingly, ~30% of the HSF direct target genes are also induced by the diauxic shift, in which glucose levels begin to be depleted. We demonstrate that HSF and Snf1 kinase interact *in vivo* and that HSF is a direct substrate for phosphorylation by Snf1 kinase *in vitro*. Furthermore, glucose starvation-dependent, but not heat shock-dependent HSF phosphorylation, and enhanced chromosomal HSF DNA binding to low affinity target promoters such as *SSA3* and *HSP30*, occurred in a Snf1-dependent manner. Consistent with a more global role for HSF and Snf1 in activating gene expression in response to changes in glucose availability, expression of a subset of HSF targets by glucose starvation was dependent on Snf1 and the HSF carboxyl-terminal activation domain.

Heat shock transcription factors (HSF)¹ play a central role in cellular homeostasis in response to a variety of stress conditions by activating gene expression. To date, most of the known HSF-regulated genes encode heat shock proteins (Hsps), which

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¹ The abbreviations used are: HSF, heat shock transcription factor; Hsp, heat shock protein; HSE, heat shock element; SC, synthetic complete medium; GST, glutathione *S*-transferase; PKA, protein kinase A; CTA, C-terminal activation domain; NTA, amino-terminal activation domain; ChIP, chromosomal immunoprecipitation.

predominantly comprise molecular chaperones involved in protein folding, stabilization, activation, trafficking, and degradation (1). The overall fundamental structure of HSF and its consensus binding site, the heat shock element (HSE) are conserved from yeast to humans (2, 3). Although yeast and *Drosophila* have a single gene encoding HSF, plants and mammals harbor multiple genes encoding HSF isoforms; for example *Arabidopsis thaliana* possesses 21 distinct HSF genes and mammals have three genes encoding the HSF isoforms, HSF1, HSF2, and HSF4 (4, 5).

HSF1, the stress-responsive mammalian HSF isoform, is activated by a wide variety of stresses that include heat shock, oxidative stress, heavy metals, infection and inflammation, and pharmacological reagents (6). In the absence of severe stress, HSF1 exists largely as an inactive monomer in the cytosol, where repressive interactions with Hsp70, Hsp90, and other co-chaperones occur. Activation of HSF1 involves multiple steps, including homotrimerization, nuclear accumulation, DNA binding, and target gene transactivation (2, 5–7). Although the mechanisms by which many diverse stresses activate HSF1 are not well understood, it has been shown that the function of redox-sensitive cysteine residues, within and adjacent to the DNA binding domain, are required to induce trimerization of HSF1 by heat shock and oxidative stress *in vitro* and *in vivo* (8). Because HSF1 is phosphorylated under normal conditions, and undergoes hyperphosphorylation in response to stress, it has been suggested that phosphorylation plays an important role in regulating HSF1 trans-activation activity. Several distinct protein kinases, and their cognate HSF1 phosphorylation sites, have been identified and suggested to play both positive and negative roles in HSF1 function and regulation (5–7, 9).

Although initial studies suggested that yeast HSF, which is essential for cell survival, is constitutively trimerized and bound to HSEs (10, 11), other studies demonstrated both constitutive and heat-inducible binding of HSF to specific HSEs in the *HSP82* promoter (12–14). Moreover, recent identification of genome-wide *Saccharomyces cerevisiae* HSF targets by chromatin immunoprecipitation, combined with DNA microarray analysis, demonstrated global heat-stimulated binding of HSF to many target genes.² Furthermore, similar to mammalian HSF1, yeast HSF is activated by multiple stresses, including heat shock, oxidative stress, and glucose starvation (16–18). *S. cerevisiae* HSF has also been shown to be hyperphosphorylated in response to stress, and it has been demonstrated that heat shock and the superoxide-generating agent menadione induce distinct patterns of HSF phosphorylation (16). The correlation between the kinetics of HSF phosphorylation and that of ex-

² J.-S. Hahn, Z. Hu, D. J. Thiele, and V. R. Iyer, submitted for publication.

TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Source
MCY1093	<i>MATa his4-539 uras3-52 lys2-801</i>	Marian Carlson
DTY125	<i>MATa his4-539 uras3-52 lys2-801 snf1Δ</i>	(17)
DTY179	<i>MATa his4-539 uras3-52 lys2-801 HSF-(1-583)</i>	(17)
W303-1A	<i>MATa ade2-1 can1-100 his3-11, 15 leu2-3,-112 trp1-1 ura3-1</i>	(58)
W303-1A <i>msn2msn4</i>	<i>MATa ade2-1 can1-100, leu2-3,-112 trp1-1 msn2-Δ3::HIS3 msn4-1::TRP1</i>	(59)
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(60)
BY4741 <i>reg1Δ</i>	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 reg1Δ::kanMX4</i>	Research Genetics
BY4741 <i>gac1Δ</i>	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gac1Δ::kanMX4</i>	Research Genetics
JHY39	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf1Δ</i>	This study
JHY31	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPN4 HSEm</i>	This study
RSY620	<i>MATa ade2-1 can1-100 his3-11 leu2-3,-112 his3 trp1-1 ura3 pep4::TRP1</i>	(61)

pression of one of the HSF target genes, *CUP1*, suggested that HSF activity might be regulated by differential phosphorylation under different stress conditions (16). On the other hand, phosphorylation of a heptapeptide region (CE2) of the yeast *Kluyveromyces lactis* HSF has been suggested to be involved in restraining HSF in an inactive state (19). Currently, no protein kinase has been identified which both phosphorylates yeast HSF and has been shown to be essential for HSF activity.

We previously demonstrated that Snf1 kinase, and its associated activation subunit Snf4, are required for the glucose starvation-induced, HSF-dependent activation of the *CUP1* metallothionein gene in *S. cerevisiae* (17). Snf1 kinase, a homologue of mammalian AMP-activated kinase, plays a central role in regulating cellular metabolism under low glucose conditions by controlling gene expression, as well as by the regulation of metabolic enzymes (20–22). Snf1 kinase forms a complex with an activating subunit Snf4, and one of the three Snf1-interacting proteins Sip1, Sip2, or Gal83, which target Snf1 to distinct subcellular locations (23, 24). Regulation of Snf1 activity involves phosphorylation of Snf1 on the activation loop by three upstream kinases (25–27), and binding of Snf4 to the Snf1 regulatory domain to relieve autoinhibition of the Snf1 catalytic domain (28). Several yeast transcription factors, including Mig1, Sip4, Cat8, and Gln3, have been shown to be regulated by Snf1 (21, 22). Phosphorylation of the Mig1 repressor by Snf1 triggers bulk relocalization of Mig1 from the nucleus to the cytoplasm, derepressing glucose-repressed genes, most of which are involved in utilization of alternative carbon sources (29). On the other hand, Snf1 activates Sip4 and Cat8 to induce expression of genes involved in gluconeogenesis and the glyoxylate cycle (30, 31) and activates Gln3 to induce nitrogen catabolite-repressible genes (32). Given previous genetic data, and the interaction of Snf1 with other yeast transcription factors, the Snf1-dependent, HSF-mediated induction of *CUP1* expression under conditions of glucose starvation suggests that HSF could be a direct substrate of the Snf1 kinase. However, this possibility has not yet been addressed. Furthermore, the requirement for Snf1 for *CUP1* activation in response to glucose starvation, but not heat stress (16, 17), suggest that Snf1 activation of HSF could provide insight into how HSF proteins in general sense multiple distinct stresses. Here we demonstrate that Snf1 interacts with HSF *in vivo* and is necessary for glucose-starvation induced HSF phosphorylation. Furthermore, Snf1 is required for more widespread activation of HSF target gene expression and for the maintenance of HSF binding to target gene chromatin *in vivo*.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Yeast strains used in this study are listed in Table I. JHY39, a *snf1Δ* derivative of BY4741, was generated by homologous recombination with a 6-kb BamHI/SphI DNA fragment from plasmid *psnf1::hisG* as described previously (17). JHY31, harboring an HSE mutation in the *RPN4* promoter, was generated by BY4741 by *in vivo* site-directed mutagenesis using oligo-

nucleotides (33). The HSE of the *RPN4* promoter (–348 TTCTA-GAAAGTTC) was mutated to TTTTAAAAGTTT (mutated nucleotides are underlined) in the chromosome of JHY31. Yeast cultures were grown in YPD medium (1% yeast extract, 2% bactopectone, 2% dextrose) or synthetic complete (SC) medium lacking appropriate amino acids or other nutrients, to maintain plasmids. Glucose concentrations were altered as specified.

Plasmids—Plasmids pYEX 4T-*SNF1* and pYEX 4T-*SNF1 K84R* were generated by cloning a 1.9-kb PCR product containing the *SNF1* open reading frame into the EcoRI and BamHI sites of modified pYEX 4T-1 (34). For pYEX 4T-*SNF1 K84R*, plasmid pRJ180 (35) was used as a PCR template. The expression vector for GST-HSF (pGEX-2T-*HSF1*) (36) was kindly provided by Dr. David Gross and the pGEX-2T-*HSF1*-(1–583), an expression vector for GST-HSF-(1–583) was generated by replacing a 0.8 kb NheI/SpeI fragment of pGEX-2T-*HSF1* with that of pHSF-(1–583) containing two stop codons after codon 583 (17). Plasmid pGEX-3X-*HSF1*-(1–300) was generated by cloning a PCR product into the SmaI/EcoRI sites of pGEX-3X. For two-hybrid analysis, the *SNF1* open reading frame was cloned into pBTM116 (37) to express a LexA-Snf1 fusion protein, and *HSF1* and *SNF4* were cloned into pACTII to generate plasmids expressing Gal4 activation domain fusion proteins (38).

RNA and Immunoblot Analysis—Total RNA was isolated from yeast cells, fractionated on 1.5% agarose-formaldehyde gels, and RNA blots were probed with ³²P-labeled DNA fragments derived from PCR-amplified DNA fragments encompassing specified open reading frames. For detection of HSF protein whole yeast cells were precipitated with a final concentration of 5% trichloroacetic acid, the pellets were washed twice with acetone, dried, resuspended in 50 μl of boiling buffer (50 mM Tris (pH. 7.5), 1 mM EDTA, and 1% SDS), and broken by vortexing in the presence of glass beads. SDS sample buffer was added to the cells extracts, and samples were boiled before loading onto 6% SDS-PAGE gels. HSF protein was detected by immunoblotting with anti-HSF antibody (39).

In Vitro Protein Kinase Assays—Yeast strain RSY620 was transformed with pYEX 4T-*SNF1* or pYEX 4T-*SNF1 K84R*, expression of GST-Snf1 was induced with 0.1 mM CuSO₄ for 3 h, and the GST fusion proteins were purified as previously described (34). Cells were disrupted with glass beads in extraction buffer (50 mM Tris-HCl (pH. 7.5), 1 mM EDTA, 4 mM MgCl₂, 5 mM dithiothreitol, 10% glycerol, and 1 M NaCl), and GST-Snf1 was purified by incubation with glutathione-agarose (Sigma). GST-Snf1 bound to glutathione-agarose was washed twice with washing buffer (50 mM Tris-HCl (pH 7.5), 4 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, and 0.5 M NaCl) and once with kinase buffer (25 mM HEPES (pH 7.5), 10 mM MgCl₂, 50 μM ATP). Kinase reactions were performed in a 20-μl reaction mixture by incubation with 5 μg of GST-HSF purified from *Escherichia coli* BL21(DE3) and 10 μCi of [³²P]ATP at room temperature for 30 min. Reactions were terminated by boiling in SDS-PAGE sample buffer.

Chromatin Immunoprecipitation—Yeast cells were grown in 50 ml of SC medium with 2% glucose to A₆₀₀ of 0.7 at 30 °C, and proteins were cross-linked to their DNA binding sites by adding formaldehyde to a final concentration of 1% to the cultures before stress, or after heat shock at 39 °C for 10 min, or after shifting to 0.05% glucose medium for 3 h. HSF-cross-linked DNA was isolated, sheared, and immunoprecipitated using anti-HSF antibody (a gift of Dr. David Gross) and purified after reversal of the cross-linking as described previously (40). For PCR analysis of immunoprecipitated DNA, ~0.1–1% of each immunoprecipitated sample was used to amplify specific promoter regions. PCR was performed for 27 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C in a 50-μl reaction with 1 μM of each primer, 2.5 mM MgCl₂, 0.1 mM

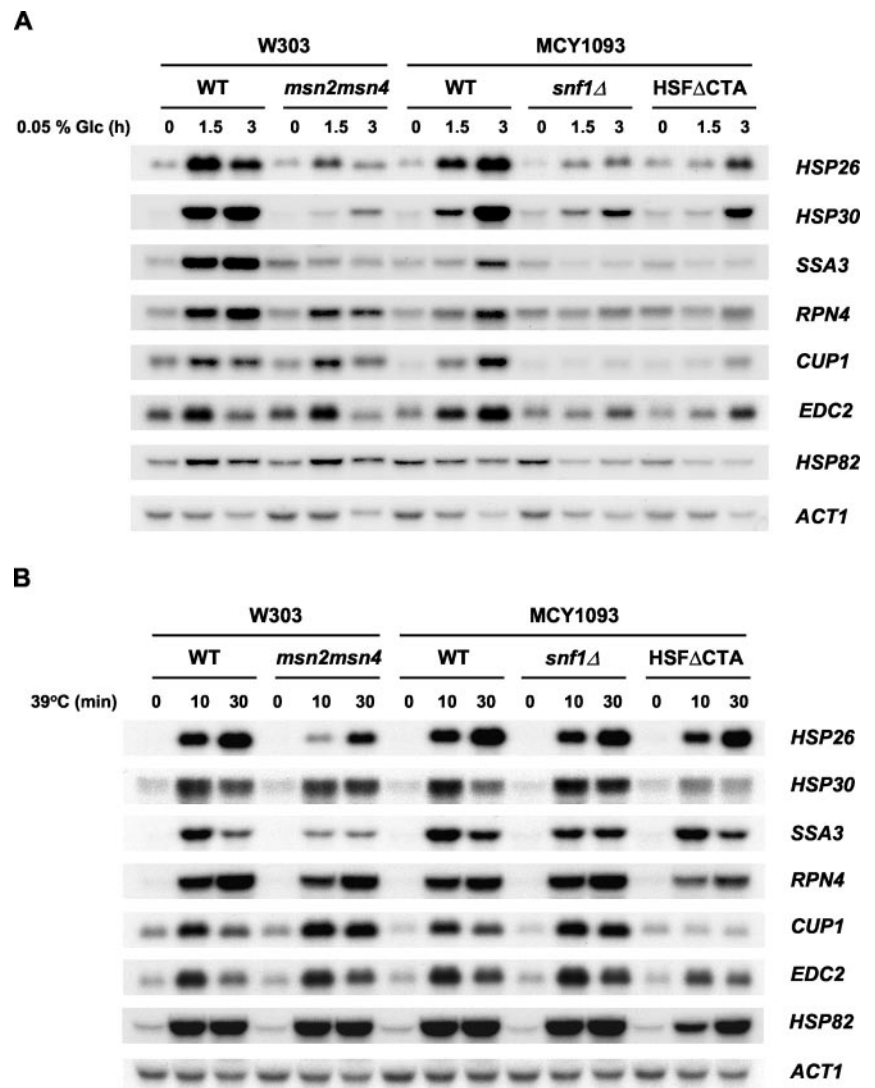


FIG. 1. Snf1-dependent induction of HSF targets in response to glucose limitation. W303-1A and the isogenic *msn2msn4* mutant strain, or MCY1093 and its *snf1*Δ or HSFΔCTA derivative were grown in SC medium containing 2% glucose and then shifted to medium with 0.05% glucose (A) or heat shocked at 39 °C (B) for the indicated times (in hours (h)). Expression levels of the indicated direct HSF target genes were detected by RNA blot analysis, with *ACT1* serving as an RNA loading control.

dNTP, and 0.1 μCi/ml [α - 32 P]dATP. PCR products were run on a 6% polyacrylamide gel and quantitated by phosphorimaging.

RESULTS

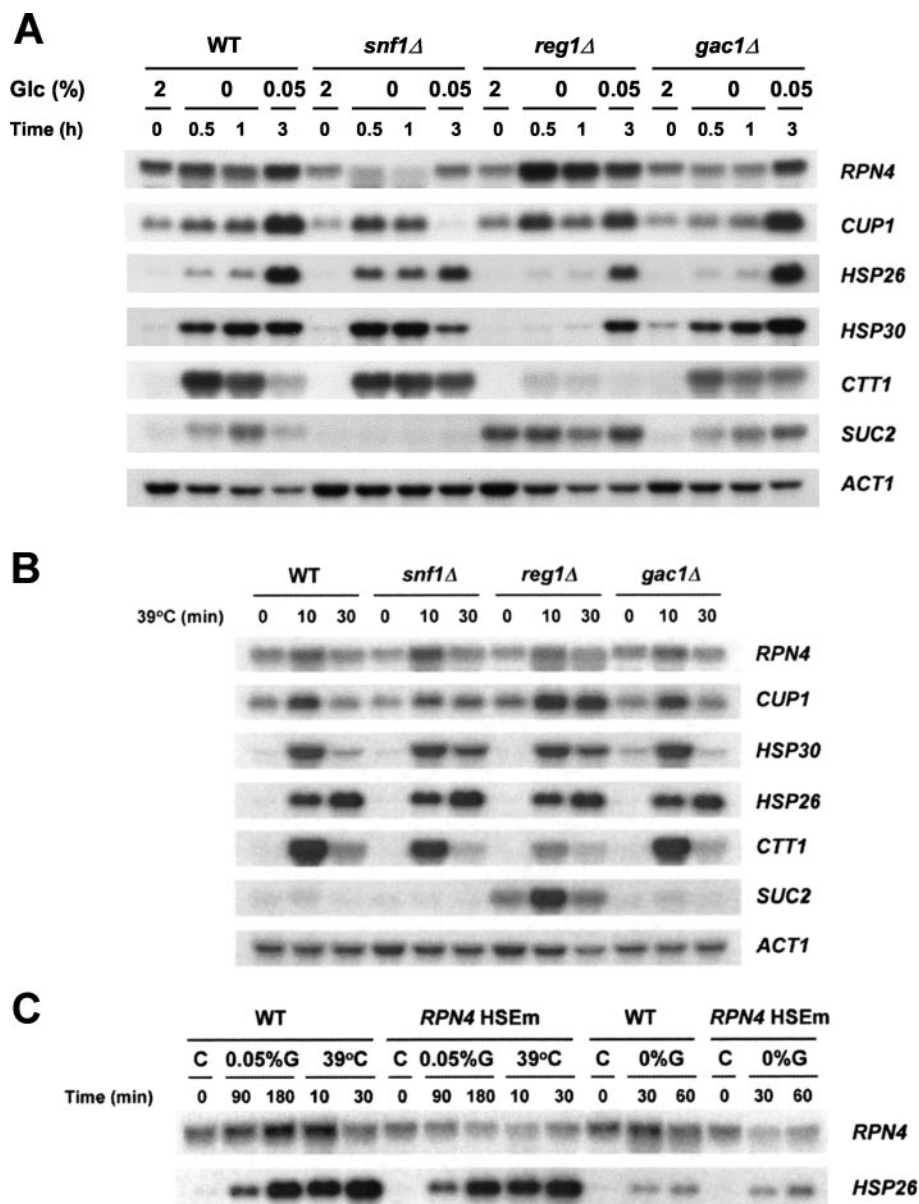
Snf1-dependent Induction of Multiple HSF Targets under Glucose Starvation—Recently we and others have identified genome-wide binding sites of the *S. cerevisiae* HSF using chromatin immunoprecipitated combined with DNA microarray analysis (41).² According to the previously published mRNA expression data (42), the large majority of the identified HSF targets that are bound by HSF *in vivo* are induced by heat shock. Interestingly, ~30% of the chromosomal HSF target genes identified by our ChIP-microarray studies are also induced during diauxic shift by more than 2-fold, in agreement with the previous reports showing activation of HSF targets such as *CUP1*, *HSP26*, and *HSP104* by glucose starvation (17, 18). We demonstrated previously that the Snf1 kinase and the Snf4 activation subunit are necessary for HSF-mediated induction of *CUP1* under glucose starvation conditions, but not in response to heat shock (17). Therefore, we examined whether the role of Snf1 can be generalized to other HSF targets, or whether this mode of activation is specific for the *CUP1* gene.

Several HSF target genes, identified previously (41) and in our genome-wide analysis, are also regulated by the general stress transcription factors Msn2/Msn4. Because Msn2 and Msn4 are negatively regulated by the cAMP-dependent kinase PKA, they are activated under low glucose conditions as well as

other stress conditions including heat shock, oxidative stress, and osmotic stress (43). Moreover, it has been shown that both HSF and Msn2/Msn4 contribute to the activation of *HSP26* and *HSP104* gene expression in response to carbon starvation (18). Therefore, we examined the effects of mutations in the *MSN2* and *MSN4* genes, as well as in a distinct HSF trans-activation domain, on the expression of HSF targets in response to glucose limitation. To test the involvement of HSF, encoded by an essential gene, we used a strain expressing HSF, which lacks the carboxyl-terminal activation domain (CTA). Unlike HSFs of other organisms, which have only a carboxyl-terminal trans-activation domain, yeast HSF possesses an additional amino-terminal activation domain (NTA), which has been shown to be involved in transient responses, whereas the CTA is involved in both transient and sustained responses (44). The HSF CTA was previously demonstrated to be responsible for activation of *CUP1*, *HSP26*, and *HSP104* in response to carbon source starvation (17, 18).

When cells grown in the presence of 2% glucose were limited for glucose by shifting to a medium containing 0.05% glucose, induction of several HSF target genes was observed after 1.5 or 3 h (Fig. 1A). For the *HSP26*, *HSP30*, and *SSA3* genes the induction was dramatically reduced in both the *msn2msn4* and HSFΔCTA strains compared with each parental wild type strain, demonstrating a contribution of both Msn2/Msn4 and HSF for their induction in response to glucose limitation. In-

FIG. 2. Differential regulation of Msn2/Msn4 and HSF by Snf1 depending on the carbon starvation conditions. BY4741 (WT) and its isogenic *snf1Δ*, *reg1Δ*, and *gac1Δ* strains, which had been grown in the presence of 2% glucose were transferred to medium without glucose or with 0.05% glucose (A) or heat shocked at 39 °C (B) for the indicated times. Levels of mRNA expression were detected by RNA blotting. C, HSE-dependent induction of *RPN4* by various stresses. BY4741 and its isogenic strain with a mutation of HSE in the *RPN4* promoter (*RPN4* HSEm) were starved with 0.05% glucose (0.05%G) or no glucose (0%G), or heat shocked at 39 °C for the indicated times. Levels of *RPN4* and *HSP26* mRNA expression were detected by RNA blotting.



duction of the HSF target genes *RPN4*, *CUP1*, *EDC2*, and *HSP82* was reduced in the HSF Δ CTA mutant, but not by the *msn2msn4* mutation, suggesting that HSF plays a more pronounced role for induction of these genes under glucose starvation conditions, with little contribution of Msn2/Msn4. However, induction of all of seven HSF targets in response to glucose deprivation was significantly compromised in *snf1Δ* compared with the isogenic wild type strain. Although induction of *HSP82* was not obvious in the wild type strain MCY1093, *HSP82* expression levels were diminished in HSF Δ CTA and *snf1Δ*, implying that HSF is necessary to maintain expression levels of *HSP82* under low glucose conditions. In contrast, deletion of *SNF1* did not affect the induction of any of the HSF targets analyzed in response to heat shock, although the induction of most of these targets was reduced in the HSF Δ CTA mutant background (Fig. 1B). The modest effect of the HSF Δ CTA allele on *HSP26* and *SSA3* induction might reflect the contribution of Msn2/Msn4 for heat shock induction of these genes and/or a reliance on the HSF NTA. Taken together these data suggest that activation of many HSF target genes is responsive to glucose starvation and is Snf1-dependent. Furthermore, these data strongly suggest that, for all of

these targets, activation by heat shock and glucose starvation is mediated by distinct signal transduction pathways.

Differential Regulation of HSF and Msn2/Msn4 by Snf1—Snf1-dependent induction of *HSP26* under glucose starvation conditions is distinct from a previous report suggesting that Snf1 negatively regulates Msn2/Msn4 under carbon starvation conditions (45). Mayordomo *et al.* (45) showed that, when yeast cells grown in rich (YPD) medium were transferred to minimal medium without any carbon source, induction of Msn2-regulated genes, including *HSP26* was increased in *snf1Δ* and decreased in *reg1Δ* strain compared with the wild type. Reg1 mediates the targeting of Glc7 phosphatase to Snf1, resulting in Snf1 inactivation. Therefore, Snf1 is constitutively active in *reg1Δ* cells even under high glucose conditions (46). To understand these apparently discordant results, we compared two different carbon starvation conditions; shift from medium with 2% glucose to medium with 0.05% glucose in which we observed a positive role for Snf1 in expression of HSF targets, or shift to a medium without any glucose (Fig. 2A).

In agreement with previous reports showing a negative role for Snf1 in Msn2 activity, induction of *HSP26* and *HSP30* was increased in *snf1Δ* and decreased in *reg1Δ* compared with the

wild type, when cells were shifted to a medium without any carbon source for 30–60 min. However, when cells were starved for glucose by shifting to medium with 0.05% glucose for 3 h, the induction levels of *HSP26* and *HSP30* were reduced in *snf1Δ* in consistent with the results shown in Fig. 1A. This positive role for Snf1 was not observed for *CTT1*, which is regulated by Msn2/Msn4 but not by HSF, consistent with Snf1 functioning through HSF. For *RPN4* and *CUP1*, which are regulated only by HSF, the negative effect of Snf1 was not observed under either carbon starvation condition. Expression of *RPN4* was reduced in *snf1Δ* under both glucose starvation conditions. Whereas, although *CUP1* induction after transfer to 0.05% glucose was clearly reduced by Snf1 deletion, induction of *CUP1* by abrupt glucose depletion was not reduced in *snf1Δ*, suggesting that some unidentified mechanisms are involved in *CUP1* induction under the latter condition. Although the basal expression of *SUC2*, which is under the control of Mig1 repressor was derepressed in *reg1Δ*, derepression of basal levels was not observed for any of the HSF targets in *reg1Δ*. A similar lack of derepression in *reg1Δ* has been observed for other Snf1-regulated genes such as genes involved in gluconeogenesis (22, 47) and *PUT1*, one of the targets of Gln3 that can be activated by Snf1. Therefore, signals in addition to the constitutive phosphorylation of Snf1 are likely to be involved in Snf1-dependent regulation of these genes. Heat shock induction of HSF targets are not affected significantly by the deletion of *SNF1* or *REG1* (Fig. 2B), confirming that the effects of Snf1 on HSF or Msn2/Msn4 are specific to glucose starvation conditions. Previously Gac1, a regulatory protein for Glc7 phosphatase, has been identified as an HSF interacting protein, and has been suggested to be involved in activation of *CUP1* by heat shock and glucose starvation (48). However, under our experimental conditions, deletion of Gac1 did not show significant effects on induction of any HSF target gene, including *CUP1*, either by heat shock or glucose starvation (Fig. 2, A and B).

To further test whether induction of *RPN4* by glucose depletion is mediated by HSF, we mutated the HSE in the *RPN4* promoter in its genomic context. In this *RPN4* HSEm strain *RPN4* expression by heat shock or glucose starvation with 0.05% glucose was not induced by stresses, confirming the role for HSF and HSE in *RPN4* induction (Fig. 2C). When cells were shifted to medium without glucose, although *RPN4* expression levels are slightly increased in the wild type, the levels were decreased in the *RPN4* HSEm. These data suggest that HSF is responsible for *RPN4* induction by both of the carbon starvation conditions, in agreement with the requirement of Snf1 for *RPN4* induction by both conditions (Fig. 2A).

Snf1-dependent Phosphorylation of HSF in Vivo and in Vitro—A well characterized function of the yeast Snf1 kinase is the regulation of transcriptional activators or repressors by direct phosphorylation (20, 21). Previous studies have demonstrated that *S. cerevisiae* HSF is hyperphosphorylated with distinct patterns of phospho-peptides in response to heat shock and oxidative stress (16). We have demonstrated the requirement for HSF, the HSE promoter binding site, and Snf1 kinase for the activation of HSF target genes in response to glucose deprivation. To begin to understand the mechanisms by which HSF and Snf1 kinase function in a glucose deprivation pathway of gene activation, we ascertained whether HSF becomes hyperphosphorylated in response to glucose deprivation.

As shown in Fig. 3A, when cells grown in 2% glucose were starved for glucose by shifting to medium with 0.05% glucose for 3 h, retardation of HSF mobility was observed with the appearance of another electrophoretic species with slower mobility. The electrophoretic mobility of HSF was similar to that for HSF found in cells grown under control conditions, when a

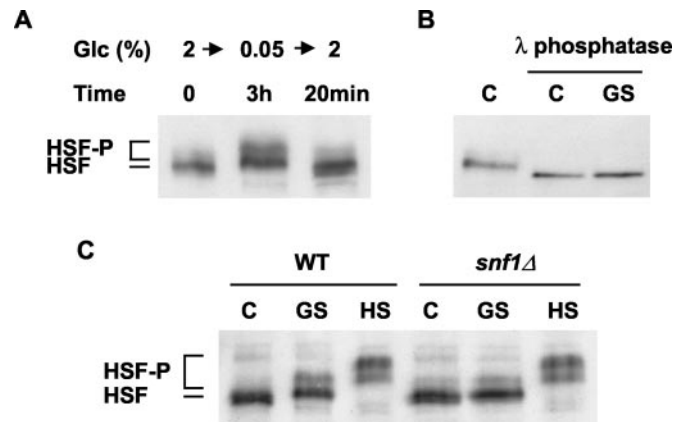


FIG. 3. Snf1-dependent phosphorylation of HSF in response to glucose deprivation. A, phosphorylation of HSF under glucose starvation conditions. Strain MCY1093, which had been grown in the presence of 2% glucose, was transferred to medium with 0.05% glucose for 3 h, cells were sampled, and glucose was added to a final concentration of 2% to the medium for 20 min. HSF was detected from total cell extracts by SDS-PAGE followed by immunoblotting with antibody against HSF. The phosphorylated forms of HSF are indicated as HSF-P. B, confirmation of HSF phosphorylation in response to glucose starvation. Cell extracts from cells grown in 2% glucose (C) or from cells grown in 0.05% glucose for 3 h (GS) were treated with λ phosphatase for 30 min, and HSF proteins were detected as described under A. C, glucose deprivation-induced HSF phosphorylation is Snf1-dependent. MCY1093 (WT) and the isogenic *snf1Δ* strain were grown in the presence of either 2% glucose (C), 0.05% glucose for 3 h (GS), or heat shocked at 39 °C for 30 min (HS), and HSF was detected and identified as described in A.

final concentration of 2% of glucose was added back to the culture for 20 min after a 3-h glucose deprivation. Consistent with the hyperphosphorylation of HSF under conditions of glucose deprivation, both the broad HSF band observed in the control conditions caused by basal phosphorylation, and the shifted band that appears under glucose starvation conditions, were collapsed down to a single sharp band after λ phosphatase treatment, in the absence, but not in the presence of phosphatase inhibitor (Fig. 3B, and data not shown). The observed phosphorylation of HSF under conditions of glucose starvation, as ascertained by the phosphatase-dependent reversal of the electrophoretic mobility shift, was abolished in the isogenic *snf1Δ* strain, whereas the well established heat shock-induced HSF electrophoretic mobility due to phosphorylation was not affected in this strain (Fig. 3C). Taken together, these results suggest that HSF- and Snf1-dependent activation of target gene expression is accompanied by Snf1-dependent HSF phosphorylation in response to glucose limitation. Furthermore, the heat shock-inducible phosphorylation of HSF is independent of Snf1, consistent with the observation that Snf1 is not required for HSF-dependent heat shock induction of any of its direct target genes.

The requirement for Snf1 for the glucose starvation-induced phosphorylation of HSF could be direct or could occur indirectly through other Snf1-dependent protein kinases. To further investigate the possibility of Snf1 as a direct HSF kinase, we examined potential interactions between Snf1 and HSF by yeast two-hybrid assays. The LexA reporter strain L40, expressing LexA-Snf1 and GAD-HSF fusion proteins, showed higher β galactosidase activity than the cells expressing either fusion alone (Fig. 4). Although an increase in the LexA-Snf1 and GAD-Snf4 interaction was detectable under glucose starvation conditions, the interaction between Snf1 and HSF was not affected by glucose concentration under these assay conditions. However, because LexA-Snf1 can be located in the nucleus irrespective of glucose status, we cannot rule out the

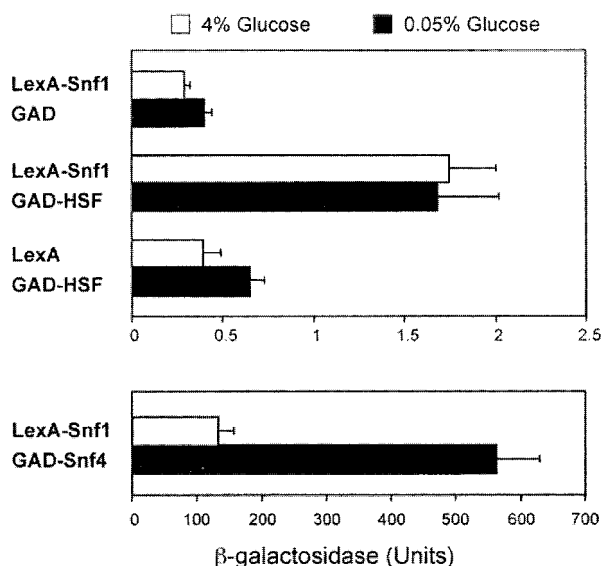


FIG. 4. Two-hybrid assay of the interaction between HSF and Snf1. *S. cerevisiae* strain L40 transformants expressing the LexA-Snf1 and GAD-HSF fusion proteins, as indicated, were grown in medium with 4% glucose and then shifted to medium with 0.05% glucose for 3 h. β -Galactosidase activities were determined from permeabilized cells. The interaction between the LexA-Snf1 and GAD-Snf4 fusion proteins was used as a positive control.

possibility that the interaction between Snf1 and HSF may be increased under low glucose conditions by translocation of Snf1 to the nucleus, where HSF is constitutively located (24, 49). The interaction between Snf1 and HSF, as detected by the yeast two-hybrid assay, supports the hypothesis that HSF might be a direct substrate of Snf1. Although attempts to demonstrate an interaction by co-immunoprecipitation were inconclusive, the rather transient association of protein kinases with their substrates may preclude the detection of these potentially weak interactions.

To further explore the potential for direct phosphorylation of HSF by Snf1, we performed *in vitro* kinase assays with GST-Snf1 purified from yeast using GST-HSF proteins purified from *E. coli* as substrates. GST-HSF fusion proteins purified from *E. coli* showed multiple bands by degradation. As shown in Fig. 5A, GST-Snf1 was autophosphorylated under these *in vitro* reaction conditions and was able to phosphorylate a full-length GST-HSF fusion protein, as well as GST fused to HSF deletion derivatives HSF-(1–583) and HSF-(1–300). Furthermore, a catalytically inactive Snf1 mutant, GST-Snf1 K84R (50), which was used in protein kinase assays at levels indistinguishable from that of wild type GST-Snf1 (Fig. 5B), was inactive for both autophosphorylation and for the phosphorylation of GST-HSF fusion proteins. These results support the hypothesis that HSF is directly phosphorylated by Snf1, and not by other protein kinases that have co-purified with GST-Snf1, unless their activation is Snf1-dependent.

Snf1 Maintains HSF DNA-binding Activity *In Vivo* under Glucose Limitation—Experiments with the *HSP82* promoter, having three HSEs (12, 13), revealed that, although HSF constitutively binds to distinct target HSEs, HSF binding to a low affinity HSE is strongly stress inducible. Furthermore, genome-wide HSF binding analyses demonstrated that HSF DNA binding activity, at many of its target loci, increases in a heat-inducible manner.² Because glucose starvation can activate the expression of many direct HSF target genes, we examined whether HSF DNA binding activity is also regulated by glucose availability. Consistent with previous observations (12, 13), the analysis of HSF binding to both the *HSP82* and *CUP1*

promoters by ChIP-PCR showed a modest increase after wild type cells were treated with a 10-min heat shock at 39 °C (Fig. 6). The increase in HSF DNA binding was more obvious for the *HSP30* and *SSA3* promoters, showing an ~8-fold increase in DNA binding activity after heat shock. These promoter-specific patterns of heat-inducible DNA binding of HSF were indistinguishable in wild type and *snf1Δ* strains, in agreement with our observations that Snf1 did not affect HSF phosphorylation or the expression of HSF targets in response to heat shock stress. In wild type cells, HSF binding to the *HSP30* and *SSA3* promoters was also increased ~2-fold by glucose starvation, although the binding to *HSP82* and *CUP1* was not changed significantly. Although the glucose starvation-inducible binding of HSF to the *HSP30* and *SSA3* promoters was subtle compared with the heat-inducible binding to these promoters, it was reproducible in three independent ChIP experiments in which the PCR products from immunoprecipitated samples were normalized with respect to the *PHO5* promoter. In contrast, HSF binding to all four promoters was decreased in the *snf1Δ* strain under glucose starvation conditions compared with the control conditions, showing a reduction of ~40–60% in binding activity. These results suggest that Snf1-dependent phosphorylation of HSF is required to maintain or increase HSF DNA binding activity for activation of the target genes in response to glucose limitation, but not in response to heat shock.

DISCUSSION

All organisms must be capable of mounting a response to a wide range of stressful conditions encountered either as a consequence of normal growth and development or by exposure to distinct environmental stresses. Heat shock transcription factor (HSF), which is highly conserved from yeast to humans, plays an important role in controlling cellular homeostasis by activating gene expression in response to many stressful conditions. The importance of HSF has been demonstrated in many organisms not only for defense against overt stress conditions but also for many crucial biological processes such as oogenesis, embryonic development, and aging (51–54). Considering the diverse roles carried out by classic heat shock proteins, and newly identified targets activated by HSF (41),² it is not surprising that HSF is activated by such a wide range of stressful conditions. The single HSF found in the baker's yeast *S. cerevisiae* is also activated by multiple stress conditions that include heat, oxidative stress, glucose starvation, pH, salicylate, and other conditions (12, 16, 17, 39). Therefore, this system provides a powerful genetic model for understanding how, like mammalian HSF1, a single HSF protein responds to multiple stresses.

In response to glucose starvation, HSF undergoes phosphorylation in a Snf1-dependent manner. The two-hybrid interaction between HSF and Snf1, and *in vitro* phosphorylation of HSF by Snf1, support the idea that HSF may be a direct substrate for phosphorylation by Snf1. Snf1-dependent induction of a subset of HSF target genes by glucose starvation suggest that phosphorylation of HSF by Snf1 is necessary for activation of HSF under glucose starvation conditions. Furthermore, ChIP-PCR analysis suggests that the DNA binding activity of HSF, especially to low affinity promoters, is stimulated under conditions of glucose starvation in a Snf1-dependent manner. The fact that Snf1-dependent phosphorylation of HSF is only required for activation of HSF by glucose starvation, but not by heat shock, suggest that HSF activity might be regulated by differential phosphorylation via different kinases in response to a variety of stress conditions. Our efforts to map the sites of Snf1 phosphorylation of HSF thus far revealed multiple sites of phosphorylation with potential redundancy.

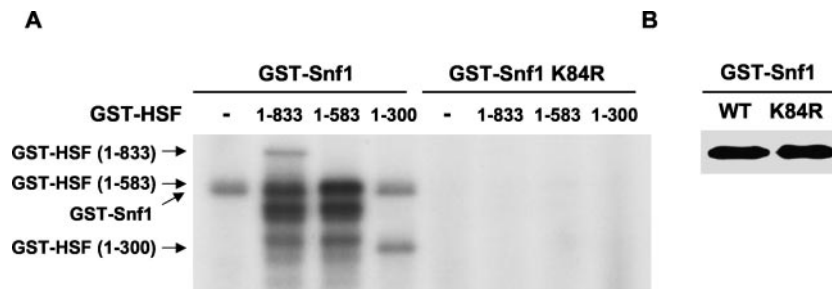


FIG. 5. **Phosphorylation of HSF by Snf1 *in vitro*.** A, GST-Snf1 or GST-Snf1 K84R proteins were purified from yeast strain RSY620 and used in *in vitro* kinase assays in the presence of GST fused to full-length HSF (*GST-HSF*), or truncated derivatives of HSF (*GST-HSF-(1-583)*), or (*GST-HSF-(1-300)*) purified from *Escherichia coli*. Reaction components were fractionated by SDS-PAGE and detected by autoradiography (B). Expression levels of GST-Snf1 and GST-Snf1 K84R used for the *in vitro* kinase assays shown in A were detected by SDS-PAGE and immunoblotting with anti-GST antibody.

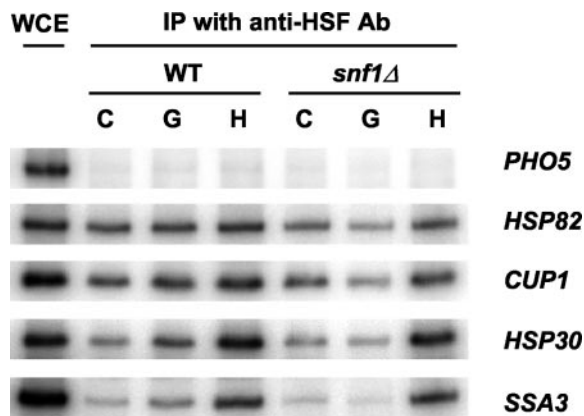


FIG. 6. **Snf1 functions in the maintenance of HSF DNA binding activity.** MCY1093 and *snf1Δ* cells were treated with formaldehyde when grown in 2% glucose (C), after 3-h glucose limitation with 0.05% glucose (G), or after a 10-min heat shock at 39 °C (H) to cross-link proteins bound to DNA. Chromatin immunoprecipitation was performed with anti-HSF antibody, and analyzed by PCR for the indicated promoters. The *PHO5* promoter, which is not bound by HSF, was used as a negative control.

Although we cannot rule out the possibility that direct sensing of heat shock or oxidative stress may also contribute in activation of yeast HSF, as demonstrated for mammalian and *Drosophila* HSF (8, 55), differential phosphorylation of HSF by stress-specific kinases might allow sophisticated levels of control of HSF activity to induce a subset of targets in response to specific stress signals. It is not clear yet why glucose starvation induces only a subset of HSF targets. However, judging from the differences in phosphorylation-mediated mobility shift of HSF by glucose starvation and heat shock, the two different forms of stress may induce different sites of phosphorylation and differential conformational changes of HSF. In addition, the contribution of other transcription factors might also confer differential regulation of HSF targets depending on the stress conditions.

The Snf1 kinase and the cAMP-dependent protein kinase, PKA, are two kinases playing opposite roles in regulating cellular growth and metabolism in response to glucose availability. Although PKA is activated by high glucose to control cellular proliferation and inhibit expression of stress responsive genes (43), Snf1 is activated by low glucose to reprogram cellular metabolism from fermentative to oxidative growth (20). The general stress transcription factors Msn2/Msn4 are negatively regulated by PKA-mediated phosphorylation, thus playing an important role in gene activation under low glucose conditions. Msn2/Msn4 are also activated by other stresses, including heat shock, oxidative stress, and osmotic stress, and induce expression of an estimated 180 genes by binding to stress-responsive elements (42, 43, 56).

Several HSF targets are also regulated by Msn2/Msn4, and the cooperative and flexible contribution of HSF and Msn2/Msn4 in expression of *HSP26* and *HSP104* by heat shock and carbon source starvation has been demonstrated previously (18, 57). We showed that the dual function of Snf1 in regulating HSF and Msn2/Msn4 can converge in the activation of genes controlled by both HSF and Msn2/Msn4. A negative role of Snf1 for expression of *HSP26* and *HSP30* by inhibition of Msn2/Msn4 was prominent under abrupt glucose depletion conditions, whereas positive effect of Snf1 via HSF made a bigger contribution when cells were starved with glucose by shifting from a medium with 2% to a medium with 0.05% glucose. The negative effect of Snf1 was not observed for expression of stress-responsive genes regulated only by HSF, such as *RPN4* and *CUP1*, supporting the previous reports showing that Snf1 negatively regulated Msn2 through controlling its cellular localization in response to glucose depletion (45). Although cAMP levels are considered as a potential signal molecule for PKA activation, the glucose signal transduction pathways regulating PKA are not well understood (43). Also, although the mammalian homologue of Snf1, AMP-activated kinase, has been known to be activated by increase in the ratio of AMP/ATP, the glucose-sensing mechanisms of Snf1 are poorly understood (20). Therefore, further examination is required to explain why inhibition of Msn2/Msn4 by Snf1 is more pronounced under one carbon starvation condition, whereas activation of HSF by Snf1 plays a major role under the other condition to activate genes regulated by both HSF and Msn2/Msn4. However, such a regulatory network mediated by Snf1 would orchestrate gene expression in a sophisticated manner by regulating both HSF and Msn2/Msn4 in response to diverse changes in carbon source status.

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