

Histone H3 Ser10 Phosphorylation-Independent Function of Snf1 and Reg1 Proteins Rescues a *gcn5*[−] Mutant in *HIS3* Expression

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Gcn5 protein is a prototypical histone acetyltransferase that controls transcription of multiple yeast genes. To identify molecular functions that act downstream of or in parallel with Gcn5 protein, we screened for suppressors that rescue the transcriptional defects of *HIS3* caused by a catalytically inactive mutant Gcn5, the E173H mutant. One bypass of Gcn5 requirement gene (*BGR*) suppressor was mapped to the *REG1* locus that encodes a semidominant mutant truncated after amino acid 740. Reg1(1-740) protein does not rescue the complete knockout of *GCN5*, nor does it suppress other *gcn5*[−] defects, including the inability to utilize nonglucose carbon sources. Reg1(1-740) enhances *HIS3* transcription while *HIS3* promoter remains hypoacetylated, indicating that a noncatalytic function of Gcn5 is targeted by this suppressor protein. Reg1 protein is a major regulator of Snf1 kinase that phosphorylates Ser10 of histone H3. However, whereas Snf1 protein is important for *HIS3* expression, replacing Ser10 of H3 with alanine or glutamate neither attenuates nor augments the *BGR* phenotypes. Overproduction of Snf1 protein also preferentially rescues the E173H allele. Biochemically, both Snf1 and Reg1(1-740) proteins copurify with Gcn5 protein. Snf1 can phosphorylate recombinant Gcn5 in vitro. Together, these data suggest that Reg1 and Snf1 proteins function in an H3 phosphorylation-independent pathway that also involves a noncatalytic role played by Gcn5 protein.

Histone acetylation is a well-studied modification of chromatin (67) and has been linked to transcriptional regulation, recombination, DNA replication, and damage repair (13). GNAT (Gcn5 protein-related *N*-acetyltransferases) and MYST (MOZ-Ybf2/Sas3-Sas2-Tip60) families of histone acetyltransferases (HATs) generate both targeted and global acetylation of the chromatin (78). Other HATs, such as TAF1 (formerly TAF_{II}250) and nuclear hormone receptor coactivators, though not belonging to either family, have also been shown to play critical chromatin-related functions via their HAT activities (78).

The *Saccharomyces cerevisiae* Gcn5 protein is the catalytic subunit of several chromatographically distinct HAT complexes, including SAGA, ADA (32), SALSA, and SLIK (70, 71, 85). SAGA is recruited to the promoter by certain transcriptional activators and causes promoter-specific nucleosomal hyperacetylation leading to transcriptional activation (4, 5, 48, 51, 72). The SAGA complex also performs HAT-independent functions, such as TATA binding protein (TBP) recruitment and histone deubiquitinylation (8, 9, 19, 24, 38, 44, 55, 75, 86). SAGA and SALSA/SLIK complexes share TBP-associated factors with TFIID (33). Low-resolution electron microscopic studies showed that the architectures of SAGA and TFIID complexes are highly similar (3, 11, 91, 103). TFIID is critical for mostly housekeeping gene expression, and the SAGA-dominated genes (~10% of the nuclear genes) are largely stress-induced and are under the coordinated control of multiple chromatin and transcriptional regulators (43).

Although the promoter-specific histone acetylation function of Gcn5 has been firmly established (48, 51), which molecular activities are modulated by histone acetylation remains an open question. The best-known molecular event triggered directly by histone acetylation is the recruitment of bromodomain-containing proteins (20, 45, 53, 62). Besides this, however, little is known as to what other functions may be triggered or antagonized by histone acetylation. Identification of mutations that suppress defects associated with histone hypoacetylation may reveal factors downstream of histone acetylation. Thus far, the only reported screen for suppressors rescuing *gcn5* null phenotypes was a multicopy suppressor hunt identifying *ARG3* (69), which is likely involved in controlling the global chromatin structure via regulating the balance of nuclear polyamine. On the other hand, Gcn5 protein is important for only a portion of yeast genes (40, 43). Suppressors that display gene specificity, instead of global effects on chromatin structure, may shed light on the molecular basis for Gcn5-mediated transcriptional activation. In our first attempt to identify the bypass of Gcn5 requirement gene (*BGR*) suppressors, we isolated one such mutation mapped to the *REG1* gene.

REG1 (also called *HEX2* and *SRN1*) was identified in several genetic screens of glucose repression and RNA processing (63, 65, 66, 96). Reg1 protein associates physically and functionally with an essential and multifunctional protein phosphatase 1, Glc7 (23, 61, 94), whose substrate specificity is apparently determined by association with different partners, including Reg1 protein. Mutations of *REG1* cause ectopic expression of several genes under repressing conditions (21, 27, 41, 64, 97, 102). Point mutations targeted at the Glc7 interaction domain of Reg1 protein derepress *ADH2* and *SUC2* (23). A similar transcriptional repression defect caused by a *glc7* mutation (T152K) can be suppressed by overexpressing Reg1

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TABLE 1. Yeast strain list

Strain	Relevant genotype	Source or reference
yMK839	<i>MATa trp1 leu2-3,112 ura3-52</i>	52
yMK842	<i>MATa trp1 leu2-3,112 ura3-52 gcn5Δ::hisG</i>	52
yMK984	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 F221A</i>	This study
yMK986	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H</i>	This study
yMK988	<i>MATa trp1 leu2-3,112 URA3::HIS3-lacZ</i>	This study
yMK995	<i>MATa trp1 leu2-3,112 URA3::HIS3-lacZ gcn5 E173H</i>	This study
yMK1055	<i>MATa trp1 leu2-3,112 URA3::HIS3-lacZ gcn5 E173H reg1::mTn</i>	This study
yMK1085	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H pMK125(CEN GCN5 TRP1)</i>	This study
YL232	<i>MATa trp1 leu2-3,112 ura3-52 snf1Δ-1::LEU2</i>	This study
YL328	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H REG1::mTn</i>	This study
YL338	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H reg1Δ::KanMX6</i>	This study
YL351	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H REG1(1-740)</i>	This study
YL352	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 F221A REG1(1-740)</i>	This study
YL375	<i>MATa trp1 leu2-3,112 ura3-52 REG1(1-740)</i>	This study
YL376	<i>MATa trp1 leu2-3,112 ura3-52 gcn5Δ::hisG REG1(1-740)</i>	This study
YL558	<i>MATa trp1 leu2-3,112 ura3-52 snf1Δ-2::TRP1</i>	This study
YL559	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H REG1::mTn snf1Δ-2::TRP1</i>	This study
YL585	<i>MATa trp1 leu2-3,112 ura3-52 GCN5 REG1(1-740) snf1Δ-2::TRP1</i>	This study
YL603	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H REG1(1-740)-myc::TRP1</i>	This study
YL610	<i>MATa trp1 leu2-3,112 ura3-52 gcn5 E173H REG1-myc::TRP1</i>	This study
JHY205	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT pJH33[CEN URA3 HTA1-HTB1 HHT2-HHF2]</i>	2
DA10	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT pQQ18[CEN LEU2 HTA1-HTB1 HHT2-HHF2]</i>	D. Almy, unpublished
YL372	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H pQQ18[CEN LEU2 HTA1-HTB1 HHT2-HHF2]</i>	This study
YL381	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H REG1(1-740) pQQ18[CEN LEU2 HTA1-HTB1 HHT2-HHF2]</i>	This study
YL407	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H REG1(1-740) pMK439S10A[CEN LEU2 HTA1-HTB1 hht2-S10A-HHF2]</i>	This study
YL408	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H REG1(1-740) pMK439S10E[CEN LEU2 HTA1-HTB1 hht2-S10E-HHF2]</i>	This study
YL409	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H pMK439S10A[CEN LEU2 HTA1-HTB1 hht2-S10A-HHF2]</i>	This study
YL410	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H pMK439S10E[CEN LEU2 HTA1-HTB1 hht2-S10E-HHF2]</i>	This study
YL457	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H pMK439S10A/S28A/S31A[CEN LEU2 HTA1-HTB1 hht2-S10A/S28A/S31A-HHF2]</i>	This study
YL458	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H REG1(1-740) pMK439S10A/S28A/S31A[CEN LEU2 HTA1-HTB1 hht2-S10A/S28A/S31A-HHF2]</i>	This study
YL459	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H pMK439S10E/S28D/S31D[CEN LEU2 HTA1-HTB1 hht2-S10E/S28D/S31D-HHF2]</i>	This study
YL460	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 hht1-hhf1::KAN hhf-2hht2::NAT hta1-htb1::HPH hta2-htb2::NAT gcn5 E173H REG1(1-740) pMK439S10E/S28D/S31D[CEN LEU2 HTA1-HTB1 hht2-S10E/S28D/S31D-HHF2]</i>	This study

protein (94). These transcriptional derepression phenotypes are likely due to the inability of Glc7 to dephosphorylate the appropriate target protein(s) and consequently the ectopic increase of protein phosphorylation. Indeed, deletion of the Snf1 protein kinase suppresses the derepression defects resulting from *reg1* or *glc7* mutations (23, 28, 42), indicating an antagonistic relationship between the Snf1 kinase and the Reg1-Glc7 phosphatase complex. Consistent with this notion, Reg1 protein interacts directly with Snf1 protein in both yeast two-hybrid assays and affinity purification (61, 79). Furthermore, a hyperactive Snf1 protein caused by *reg1Δ* rescues the Spt⁻ phenotypes of *spt21Δ* cells (39). Curiously, the interaction between Reg1 protein and Snf1 protein, at least within the yeast two-hybrid context, is enhanced in glucose starvation conditions (61), raising the possibility that Reg1 protein may have a positive role in Snf1 protein action under certain conditions.

Snf1 protein acts as a cellular fuel gauge controlling re-

sponses to nutritional crises (37). The animal homologues of Snf1 protein are activated by AMP and are referred to as AMP-activated protein kinases. In plants, Snf1 protein-related kinases (SnRKs) fall into three large families, SnRK1, SnRK2, and SnRK3 (36). Snf1 protein, AMP-activated protein kinases, and SnRKs are the catalytic α subunits of a trimeric complex composed of a scaffold β protein and a regulatory γ subunit. In addition to bridging the α and γ subunits, the β protein contributes to substrate selection as well. The γ subunit of the yeast Snf1 complex is encoded by *SNF4* (14). At least three yeast genes encode the β subunits (26, 104). Snf1 protein plays critical roles in controlling transcription of carbohydrate transporter and metabolism genes (80). Overexpression of Snf1 protein also causes early aging, increased rRNA recombination, and loss of rRNA locus silencing (56), a set of functions reportedly linked to histone H3 hyperphosphorylation. Indeed, several proteins can be phosphorylated by Snf1 protein in

TABLE 2. Plasmid construct list

Plasmid	Description	Source or reference
pMK125	pRS414- <i>GCN5</i>	52
pMK284	Integration construct for introducing point mutations to <i>GCN5</i>	This study
pMK284E173H	Integration construct for introducing E173H to <i>GCN5</i>	This study
pMK284F221A	Integration construct for introducing F221A to <i>GCN5</i>	This study
pMK334	Integration construct for introducing <i>HIS3-lacZ</i> reporter to <i>URA3</i> locus	This study
pMK449	pBluescriptKS- <i>SNF1</i>	This study
pMK453	<i>snf1Δ::LEU2</i> disruptor	This study
pKD97	pRS316- <i>HA-reg1Δ6</i>	23
pYL31	pRS316- <i>HA-REG1(1-740)</i>	This study
pYL35	Integration construct for introducing <i>REG1(1-740)</i> mutation	This study
pYL41	YEplac112- <i>SNF1</i>	This study
pYL42	pYEX-4T-GST-Snf1	35
pYL44	pYEX-4T-GST	A. Acharya, unpublished
pYL45	<i>snf1Δ::TRP1</i> disruptor	This study
pYL54	pYEX-4T-Gcn5-TAP	This study
pYL67	8 × <i>Myc::TRP1</i> for tagging proteins with 8 Myc repeats	This study
pQQ18	pRS315- <i>HTA1-HTB1 HHT2-HHF2</i>	2
pJH33	pRS316- <i>HTA1-HTB1 HHT2-HHF2</i>	2
pMK439S10A	pQQ18 with an H3 S10A mutation	This study
pMK439S10E	pQQ18 with an H3 S10E mutation	This study
pMK439S10A/S28A/S31A	pQQ18 with an H3 S10A/S28A/S31A mutation	This study
pMK439S10E/S28D/S31D	pQQ18 with an H3 S10E/S28D/S31D mutation	This study
pMK515	pET21-6xHis-Gcn5 protein	This study
pMK547Gcn5	3xHA-Gcn5 overexpression	This study

response to glucose starvation, including Reg1 protein (79), Mig1 (92), and histone H3 (60). The histone H3 phosphorylation activity of Snf1 protein has been linked directly to transcriptional activation and TBP recruitment (58, 59). Ser10 phosphorylation facilitates acetylation by increasing the affinity between Gcn5 protein and H3 (15, 18, 60). Both modifications are important for the expression of the *INO1* gene in yeast (59, 60). In addition, genetic interactions between Snf1 protein and Srb/mediator proteins (49, 84) and TBP (83) were reported. Whether these general transcriptional factors can be phosphorylated by Snf1 protein is unclear.

In this work, evidence that a gain-of-function *BGR* allele for Reg1 protein likely adopts a novel function in facilitating transcription of *HIS3* is presented. This function appears to require a functional Snf1 kinase. However, H3 phosphorylation does not play a critical role for the suppression, nor is it important for normal *HIS3* activation. A unique allele specificity for a particular mutant Gcn5 protein is shared by the Reg1 suppressor and overproduction of Snf1 protein. Indeed, both Snf1 and Reg1 suppressor can be copurified with Gcn5 from yeast, linking these three proteins functionally and physically.

MATERIALS AND METHODS

Yeast strains, plasmids, and genetic methods. Yeast strains used in this work are listed in Table 1. All genetic methods were according to reference 81. Yeast transformation was done with the lithium acetate method (29). Plasmids used in this work are listed in Table 2.

To introduce *gcn5* point mutations into the genome, the BamHI-HindIII fragment from wild-type or mutant *GCN5* was inserted into the same sites of YIplac211 (30) to generate pMK284. Constructs pMK284E173H and pMK284F221A were linearized with NgoMIV and transformed into yeast. Integration results in two copies of *GCN5* separated by the YIplac211 sequence containing a *URA3* marker. 5-Fluoroorotic acid (5-FOA) selection and genomic PCR were used to obtain and verify the desired E173H and F221A mutations.

The *HIS3-lacZ* reporter was introduced to yeast by transforming the StuI-linearized pMK334 that generates *URA⁺* integrants. pMK334 was constructed by

inserting the EcoRI-DraI *lacZ* fragment of pLKC482 (90) into the EcoRI-HindIII sites of YIplac211, resulting in pMK333. An EcoRI-BglII fragment containing the *HIS3* promoter was isolated from pMK231 where a BglII site was introduced at the 5' end of *HIS3* open reading frame (ORF) and inserted into the EcoRI-BamHI sites of pMK333. A unique StuI site within the *URA3* gene was used for integrative transformation. All subsequent integrants were grown in the absence of uracil to maintain the integrated sequence.

To knock out the *SNF1* gene, two disruptors were constructed. *snf1Δ-1::LEU2* was generated by two-step subcloning. First, an ApaLI-HindIII fragment upstream of the *SNF1* ORF was inserted into the XbaI-HindIII sites of pJJ252 (47) to create pMK452. The 3' flanking region of the *SNF1* gene, an HpaI-SacI fragment obtained by PCR, was inserted into the BamHI-SacI sites of pMK452 to obtain pMK453. In the other disruptor (pYL45, *snf1Δ-2::TRP1*), the PstI-HindIII fragment of *SNF1* was first inserted into pBluescript KS+ (pMK449). The AfIII-BglII 200-bp fragment corresponding to amino acids 109 through 176 of *SNF1* in pMK449 was replaced with the EcoRI-BglII fragment of pJJ248 containing the *TRP1* gene (47). To create *snf1* deletion strains, the HindIII-BamHI fragment of pMK453 or the EcoRI-BamHI fragment of pYL45 was obtained by restriction digestion before yeast transformation.

To introduce the *REG1(1-740)* allele, plasmid pYL31 was constructed by replacing the ClaI-BglIII fragment of pKD97 (23) with a ClaI-XhoI-digested PCR product that contains the open reading frame of *REG1* up to amino acid residue 740 followed immediately by a stop codon. The ClaI-KpnI fragment of pYL31 was cloned into HindIII-KpnI sites of YIplac211 to obtain pYL35. To replace the entire *REG1* ORF with *REG1(1-740)*, pYL35 was linearized by SnaBI and integrated into the *REG1* locus by homologous recombination. The correct transformants were subjected to 5-FOA selection. Genomic PCR confirmed the correct genotype.

The *reg1Δ* strains were generated by introducing a PCR fragment containing the KanMX6 cassette flanked by *REG1* sequences outside the ORF (10, 99). G418-resistant transformants were examined by genomic PCR to confirm the *reg1Δ* genotype.

To create and test histone H3 mutations, strain JHY205 (2) was first made *HIS3⁺* by replacing the *his3Δ1* allele with the BamHI fragment of pJJ217 (47) that contains the entire *HIS3* gene, resulting in yDA10. Each histone H3 mutation was generated by the Quikchange method (Stratagene), using pJH33 as the template. All mutations were confirmed by sequencing.

The 2- μ m *SNF1* construct pYL41 was created by cloning the BamHI-PstI fragment containing the entire transcription unit of *SNF1* into EcoRI-PstI sites of YEplac112 (30). Deletion of the general control-responsive element (GCRC) was as described previously (51).

pMK547Gcn5 with an N-terminal hemagglutinin (HA) tag was created by

cotransforming XbaI-linearized pMK547, derived from pAB8 with the Gal4 DNA binding domain deleted (34), and a PCR-amplified *GCN5* open reading frame. The Gcn5-TAP fusion construct (pYL54) was generated by a strategy essentially equivalent to QuikChange mutagenesis protocol (Roche) except that the mutagenic primers were PCR-amplified TAP sequence (74) flanked with sequences around the stop codon of *GCN5*. pMK144 (52) was the template for mutagenesis and insertion of the TAP sequence. pYL67, a plasmid derived from pBS1479 (74) by replacing the TAP sequence with eight Myc repeats, was severed as PCR template to amplify the *Myc::TRP1* cassette with flanking sequence correlated with residue 740 or the stop codon of *REG1*. Gel-purified PCR products were transformed into yeast cells to generate Reg1-Myc fusions.

HAT and kinase assays. Gcn5 protein amino acids 19 to 348 lacking the bromodomain were cloned into pET21a and expressed as a His-tagged protein (pMK515). The desired point mutations were generated by the Quikchange method (Stratagene) and verified by sequencing. The recombinant protein was induced in the BL21 strain by adding 1 mM (final concentration) IPTG (isopropyl- β -D-thiogalactopyranoside) when cell culture reached an optical density at 600 nm (OD_{600}) of 0.5/ml. Cell cultures were grown at 37°C for 3 h. Extraction and protein affinity purification were done according to reference 50.

Kinase assays were done with the above Gcn5 protein incubated with glutathione *S*-transferase (GST)-Snf1 (wild-type or K84R) expressed and purified from yeast according to reference 35. The GST-SNF1 constructs were kindly provided by D. Thiele (Duke University).

Suppressor screening. The yeast genomic DNA library (#21) containing the *mTn-lacZ/LEU2* intervening sequence was provided by M. Snyder (Yale University) (77). The DNA was prepared by cesium chloride gradient and digested by NotI before transforming into yMK995. Ten micrograms of the library DNA was digested and isolated by phenol-chloroform extraction and ethanol precipitation. Approximately 26,000 *LEU*⁺ transformants were replica plated to synthetic complete (SC)-His medium containing 20 mM 3-amino-1,2,4-triazole (3-AT) and incubated at 37°C for 3 to 5 days. 3-AT-resistant colonies were further transferred to nitrocellulose membranes, and the *lacZ* level was tested according to reference 1. Colonies that showed blue color on the *lacZ* filter assays were grown in SC-Leu medium overnight and transferred to yeast extract-peptone-dextrose (YPD) (representing the repressed condition) or synthetic minimal medium (SD) containing 20 mM 3-AT for 4 h. Yeast cells (20 ml; OD_{600} of 0.1/ml) were then harvested by centrifugation (10,000 \times g for 5 min at 4°C), washed, and suspended in extraction buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM MgCl₂, 10 mM Tris HCl, pH 7.4, 5 mM EDTA, Complete protease inhibitor cocktail [Roche]). Whole-cell extracts were prepared by vigorous agitation with glass beads using a bead beater (Biospec Products). β -Galactosidase activity was quantified according to reference 1. One clone, renamed yMK1055 henceforth, repetitively showed elevated *lacZ* expression in response to amino acid starvation and was further studied. yMK1055 was backcrossed to yMK1075 before 3-AT tests. To verify that a single *mTn* insertion event was responsible for the *BGR* phenotypes, yMK1055 was crossed to yMK1085. The diploid strain was subjected to sporulation and tetrad dissection; all *trp*⁻ segregants were tested for cosegregation of 3-AT resistance and leucine prototrophy. Recommended procedures were employed to map the integration site of the *mTn-lacZ* fragment (http://ygac.med.yale.edu/mtn/insertion_libraries.stm). Namely, yeast genomic DNA was isolated, digested by EcoRI, and subjected to intramolecular ligation prior to bacterial transformation. Plasmid DNA was isolated from *Escherichia coli* cells and sequenced across the junction between *REG1* and *mTn-lacZ/LEU2* using a primer specific to *lacZ*.

Northern analyses. Yeast cells were grown in appropriate selection media until the OD_{600} reached 0.5. Cells were then pelleted by centrifugation (5,000 \times g, 5 min, 4°C) and transferred to either YPD (for basal expression) or SD supplemented with required nutrients and 40 mM 3-AT (for induced expression). Cell suspensions were further incubated at 37°C for 2 to 3 h before harvesting for RNA preparation. Although these relatively harsh conditions for induction were not essential, such treatment generally generated more consistent results in *HIS3* activation in our strain background. Procedures for RNA preparation and Northern blot hybridization were described previously (52).

Interaction between Gcn5 and Snf1. To test the interaction between Gcn5 and Snf1 proteins, a GST-Snf1 expression construct (35) or just GST (pYL44) was transformed to the strain carrying pMK547Gcn5. Purification of GST-Snf1 was as described previously (35). Glutathione Sepharose 4B (30 μ l; Amersham) was added to whole-cell extracts purified from 1.5×10^9 cells and incubated at 4°C for 3 h under constant rocking. Beads were pelleted and washed twice with HEMGT buffer (25 mM HEPES, pH 7.9, 12.5 mM MgCl₂, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 \times Complete protease inhibitor cocktail [Roche]) followed by two more washes with HEMGT buffer containing 300 or 500 mM NaCl. The bound frac-

tions were eluted by sodium dodecyl sulfate (SDS) loading buffer and resolved by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The copurified HA-Gcn5 protein was detected by anti-HA antibodies (12CA5; Roche).

For Gcn5-Reg1(1-740) copurification, whole-cell extracts from cells carrying pYL54 and C-terminally Myc-tagged Reg1 or Reg1(1-740) protein were prepared with the bead-beating method in FA lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 \times Complete protease inhibitor cocktail [Roche]). Lysates from 3×10^9 cells were incubated with 30 μ l of immunoglobulin G Sepharose 6 (Amersham) for 2 h at 4°C. After three washes with FA lysis buffer, the beads were boiled in SDS loading buffer and resolved by 8% SDS-PAGE. Western blots were conducted with an anti-c-Myc antibody (Roche).

RESULTS

Screening and identification of a BGR suppressor. To screen for extragenic suppressors that rescue transcriptional defects caused by loss-of-function mutations of Gcn5 protein, we introduced a single mutation to the catalytic domain of Gcn5 protein. This mutation, E173H, is a Glu-to-His mutation at residue 173. Since Gcn5 protein participates in more than one complex, the use of this mutant likely maintains the integral architecture of these complexes (103). Previously, an E173Q mutation was shown by others to drastically reduce the *in vitro* and *in vivo* activity of Gcn5 protein (54, 73, 93). However, this E173Q mutant in our hands maintained significant activities in *HIS3* expression after it was integrated back to the native *GCN5* locus (data not shown). We thus designed a Glu-to-His mutation. With the slight positive charge of histidine under physiological pH, a more drastic reduction of the catalytic power of Gcn5 protein was expected (54, 89). The HAT activity of a bacterially expressed E173H mutant was tested using chicken histones as the substrates. As predicted, this mutation significantly reduced the *in vitro* HAT activity of Gcn5 protein (Fig. 1A). To test *in vivo* functions, the E173H allele was integrated to the native *GCN5* locus to replace the wild-type allele. In parallel, another well-characterized F221A allele (52) was integrated in the same manner. Both alleles were controlled by the native *GCN5 cis* elements. Yeast strains bearing the wild-type, complete knockout, F221A, or E173H allele of *GCN5* were then tested for responses to amino acid starvation. Each strain was patched to YPD medium and then replica plated to synthetic complete medium lacking histidine and supplemented with various concentrations of 3-AT, a competitive inhibitor of the His3 protein. Very minor growth defects were seen in *gcn5*⁻ strains when assayed at 30°C in medium supplemented with 3-AT (Fig. 1B). However, when these cells were incubated at 37°C, 3-AT induced obvious growth defects of all three *gcn5*⁻ strains. None of these cells were temperature sensitive (compare growth on YPD and SC-His without 3-AT). The clear growth defects of *gcn5*⁻ cells provide a platform for suppressor screening.

We further modified the E173H mutant strain by introducing a *HIS3-lacZ* reporter to the *ura3-52* locus. Insertion of *HIS3-lacZ* did not change the cellular sensitivity to 3-AT (Fig. 1B, bottom two patches). This *lacZ* reporter, under the control of the *HIS3* promoter, was also activated by amino acid starvation (Fig. 1C) and hence offered a convenient means to verify the 3-AT-resistant suppressor phenotypes.

To identify suppressors, we used a minitransposon (*mTn*)-based mutagenesis approach (77). In this method, the *mTn-lacZ/LEU2* sequence was integrated into a yeast genomic DNA

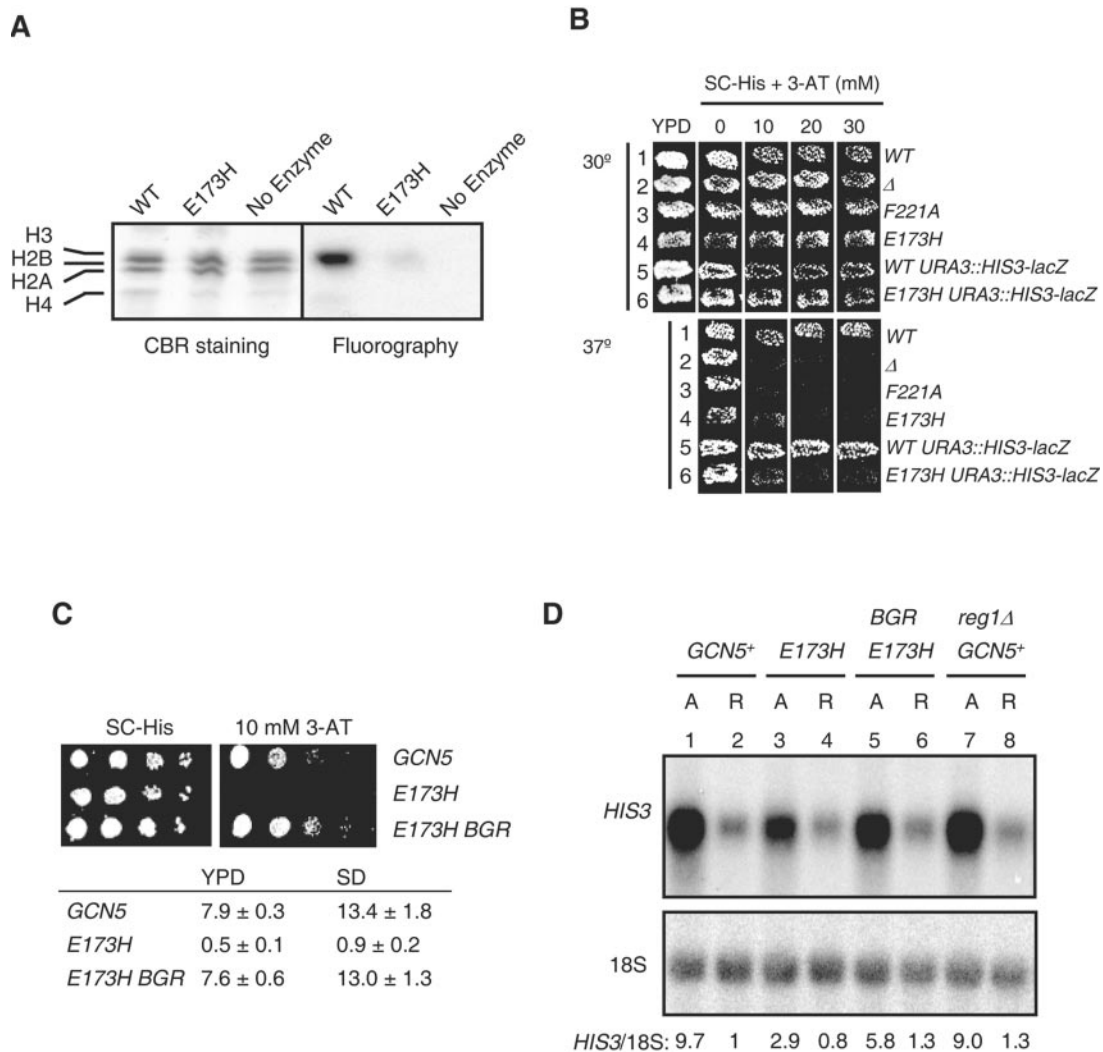


FIG. 1. Identification of a *BGR* suppressor rescuing the *gcn5* E173H mutant. (A) In vitro HAT assays. Chicken core histones were acetylated by recombinant Gcn5 proteins, and the ³H-labeled acetylated histone was detected by fluorography (right panel). CBR, Coomassie blue R250. (B) Temperature-dependent hypersensitivity to 3-AT is generated by three *gcn5*⁻ alleles. The E173H and F221A alleles were introduced to the native *GCN5* locus under the control of its own *cis* elements. Each strain was patched to YPD and grown at 30°C for 2 days. Cells were then replica plated to SC-His medium supplemented with various concentrations of 3-AT and incubated at 30 or 37°C for 3 to 4 days. Relevant genotypes are listed on the right. (C) The *BGR* suppressor rescues both 3-AT hypersensitivity and *HIS3-lacZ* expression. Both plates were from cultures grown at 37°C. The β-galactosidase activities (U/mg of total proteins/min) were measured from cultures grown in YPD or SD (minimal) medium to early log phase. Errors represent variation from two independent cultures of each strain. (D) Northern blot hybridization. Log-phase cells were harvested from rich or minimal medium supplemented with 20 mM 3-AT before RNA preparation and hybridization. 18S rRNA was used as the internal control. The ratio of *HIS3* to 18S rRNA was measured and normalized to the basal expression of a *GCN5*⁺ strain. A, activated; R, repressed. Figures of gel staining, fluorography, and culture plates were scanned with a flatbed scanner and acquired by Photoshop 7.0.

library via transposition. Yeast DNA fragments along with the interrupting sequence were excised from the plasmid pool and transformed into yeast. Each *mTn* sequence integrated to the chromatin via homologous recombination between the flanking yeast sequence and the corresponding genomic locus. *LEU*⁺ transformants were replica plated to 20 mM 3-AT medium and grown at 37°C. All 3-AT-resistant clones were then screened for increased expression of β-galactosidase induced by amino acid starvation. From approximately 26,000 *LEU*⁺ transformants, we identified 1 such colony (Fig. 1C). Northern data clearly showed that the *HIS3* expression was upregulated in this suppressor strain compared with the parental *gcn5*

E173H cells (Fig. 1D). Similar complementation in transcription was seen in *HIS1*, *HIS6* (not shown), and *HIS4* (Fig. 2C) as well. Genetic assays showed that a single *mTn* insertion event was responsible for the suppression phenotypes (data not shown and see Materials and Methods).

To map the mutation, we rescued and cloned the *mTn* insertion along with the flanking yeast sequences (see Materials and Methods). DNA sequencing across the junction revealed that the mutagenic fragment had inserted to the coding region of *REG1* (Fig. 2A), resulting in in-frame fusion of *lacZ* to residue 740 of Reg1 protein. While the expression of the Reg1-*mTn-lacZ* fusion protein may have contributed to some β-ga-

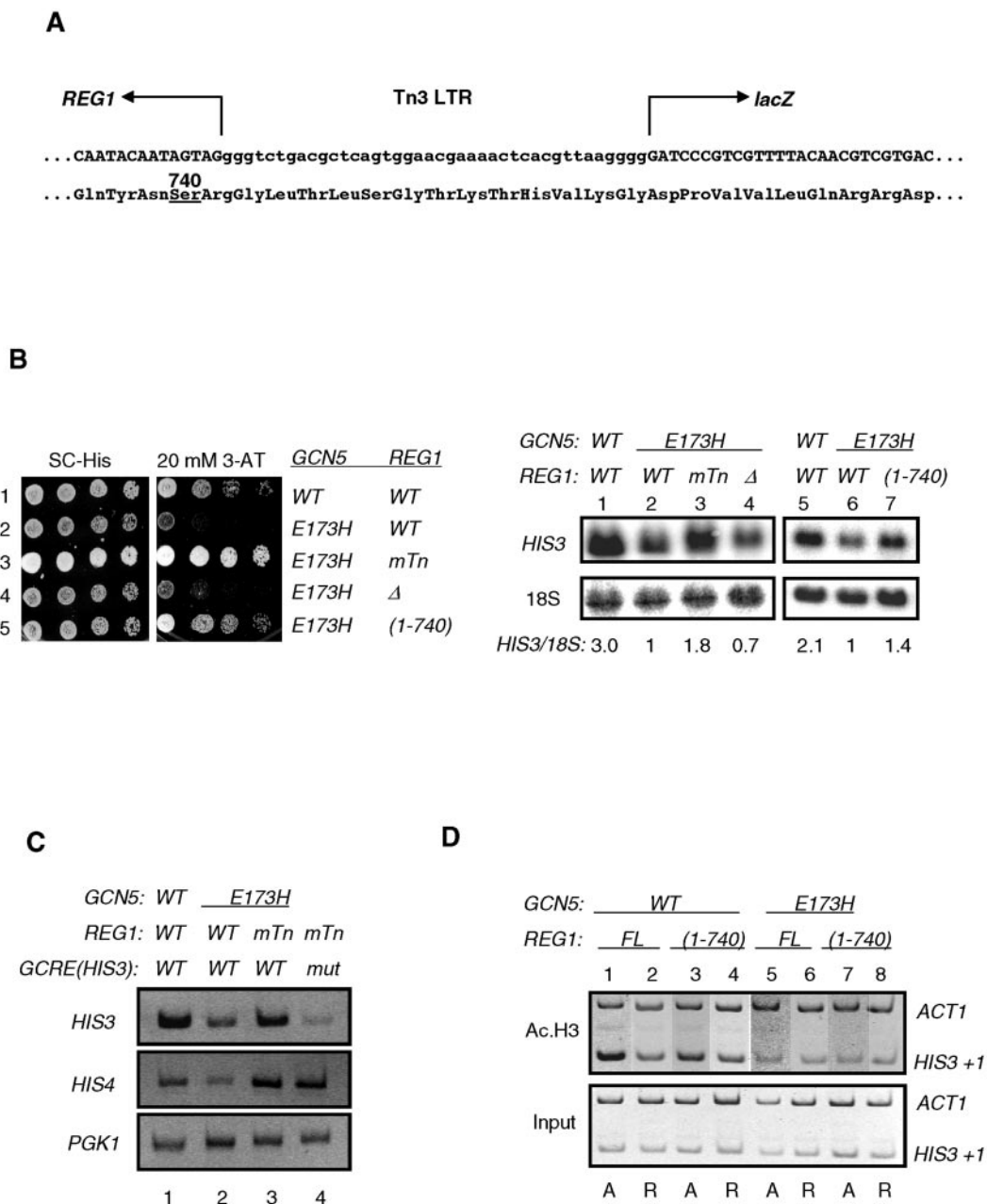


FIG. 2. Characterization of the Reg1(1-740) BGR suppressor. (A) DNA and protein sequences across the *mTn-lacZ* integration site. Insertion of the *mTn-lacZ* fragment at nucleotide 2122 results in an in-frame fusion between the Reg1 protein and the Tn3 long terminal repeat (LTR; lowercase) and the *lacZ* gene. Ser740 of Reg1 protein is marked. The nucleotide and amino acid residue numbers are relative to the start codon of the *REG1* open reading frame. (B) Reg1(1-740) protein is essential and sufficient for the BGR phenotypes. The left panel shows 3-AT tests (37°C) of isogenic strains bearing different alleles of *REG1*. The right panel shows Northern hybridization results. All samples were obtained from induced conditions (see legend to Fig. 1D). The *HIS3*/18S rRNA ratio of each sample was calculated and then normalized to that of the *gcn5* E173H strain (lane 2 or 6). (C) Reg1(1-740) protein-mediated suppression requires the Gcn4 activator binding site, GCRE. Shown are reverse transcription-PCR results. *PGK1* is an internal control. (D) Promoter H3 hypoacetylation is not affected by the Reg1(1-740) suppressor. Shown are quantitative PCR results of chromatin immunoprecipitation using an antibody against H3 acetylated at Lys9/14. *ACT1* open reading frame was used as the internal control.

lactosidase activity shown in Fig. 1C, the *HIS3* transcript quantification results (Fig. 1D) unequivocally demonstrated the rescue of *gcn5*⁻ defects. Nonetheless, since the in-frame fusion of *lacZ* added a large mass to the truncated Reg1 protein, we were curious whether the β-galactosidase fusion was necessary

for the suppression. By integrative transformation, we replaced the chromosomal copy of the native *REG1* with one that is truncated after residue 740 (without the *lacZ* fusion) and tested whether this “clean” *REG1*(1-740) allele was able to suppress the *gcn5* E173H mutation. Figure 2B shows that with

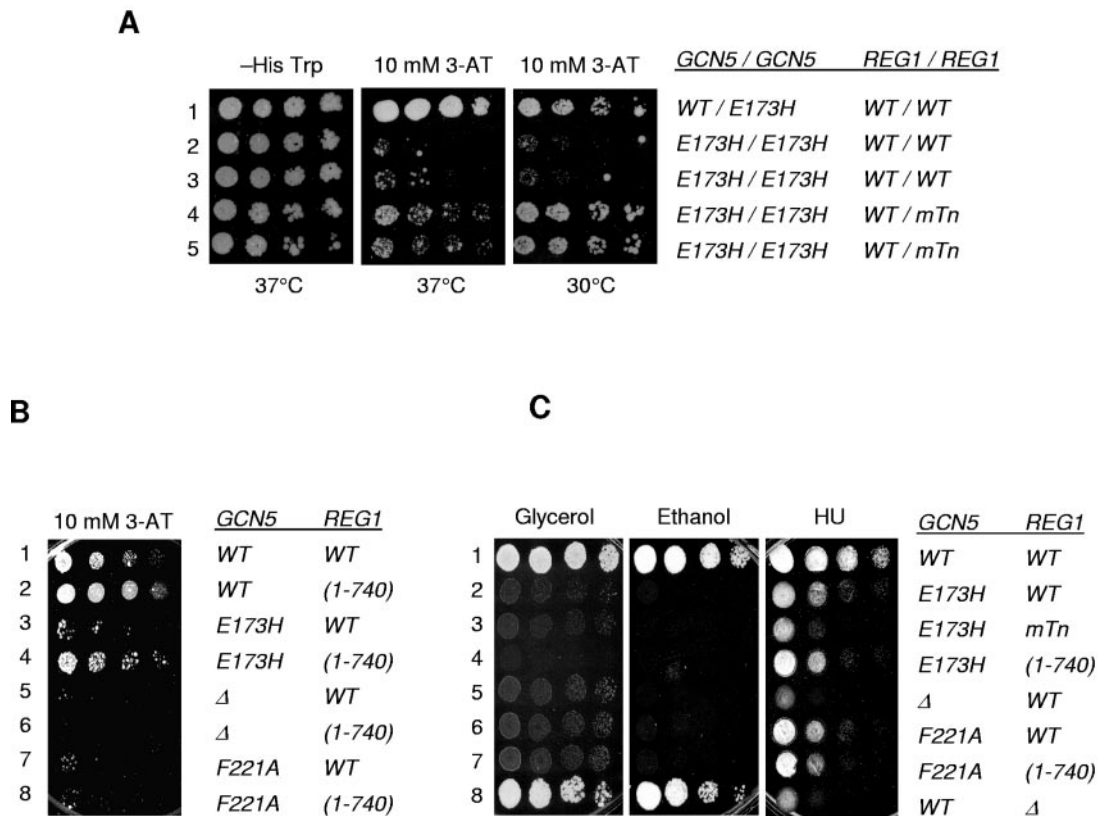


FIG. 3. The *BGR* suppressor is semidominant and selectively rescues the *E173H* defects of the *GCN* pathway. (A) Semidominant features of the *REG1(1-740)* allele. Diploid strains bearing different combinations of *GCN5* or *REG1* alleles were tested for their resistance to 3-AT at 30 or 37°C. (B) Allele specificity of the suppression. The *REG1(1-740)* allele was integrated to the chromosome to replace the wild-type *REG1* gene in different *gcn5*⁻ strains. Resultant strains were then spotted to 3-AT medium and grown at 37°C. (C) Growth defects in different conditions caused by *gcn5*⁻ mutations are not affected by the *Reg1(1-740)* protein. Indicated strains were grown to log phase in YPD, spotted to yeast extract-peptone-glycerol, yeast extract-peptone-ethanol, or YPD containing 100 mM hydroxyurea (HU), and incubated at 30°C for 3 to 4 days.

a truncated *Reg1(1-740)* protein, the *gcn5 E173H* cells also exhibited significant resistance to 3-AT (Fig. 2B, row 5) and restoration of *HIS3* expression (Fig. 2B, lane 7), although the original *mTn-lacZ* insertion consistently showed better growth than *Reg1(1-740)*. The in-frame fusion of β-galactosidase enhanced but was not essential for suppression efficacy.

We further deleted the *REG1* gene and found that the suppression phenotypes were lost (Fig. 2B, left panel, row 4, and right panel, lane 4). Interestingly, in the presence of a functional *Gcn5* protein, deleting the *REG1* gene does not seem to affect *HIS3* expression (Fig. 1D, lanes 7 and 8). Together, these results showed that a *Reg1(1-740)* truncated protein is essential and sufficient for suppressing the *gcn5 E173H* mutation in *HIS3* transcription.

Gcn5, in the context of SAGA complex, is recruited to the *HIS3* promoter by the transcriptional activator *Gcn4* that binds the cognate *cis* element, GCRC (51). To test whether the *Reg1(1-740)* suppressor exerted its function via *Gcn4*-GCRC or a novel *Gcn4*-independent mechanism, we replaced the GCRC 5' to the *HIS3* gene with an irrelevant sequence (51) and determined whether the suppression was affected. Comparison of mRNA transcribed from the GCRC-less *HIS3* with the wild-type *HIS4* control clearly showed that the GCRC was essential for *Reg1(1-740)*-mediated suppression (Fig. 2C), in-

dicating that *Reg1(1-740)* protein modulates an activity downstream of the normal *Gcn4* functions.

One possible mechanism for the observed suppression is restoration of the HAT activity of the *Gcn5 E173H* mutant protein. To see if this was the case, we conducted chromatin immunoprecipitation using an antibody against histone H3 acetylated at Lys9 and/or 14 (52). Figure 2D, lane 1, shows the expected hyperacetylation at the +1 nucleosome of *HIS3* in the presence of a wild-type *Gcn5* protein (51). The TATA element and the transcription initiation sites are within the +1 nucleosome. Promoter hyperacetylation was lost in the *E173H* background (Fig. 2D, lane 5). When *REG1* was replaced with the *REG1(1-740)* suppressor allele, H3 remained hypoacetylated at the *HIS3* promoter (Fig. 2D, lane 7), suggesting that the canonical nucleosomal H3 acetylation by the *Gcn5* acetyltransferase was not affected in the *REG1(1-740)* background.

***Reg1(1-740)* preferentially rescues the *E173H* allele of *gcn5* in a semidominant manner.** To characterize the *Reg1(1-740)* suppressor further, we first tested whether this allele was dominant or recessive. Figure 3A shows that the *REG1*⁺/*REG1(1-740)* heterozygote retained a growth advantage over the parental *gcn5 E173H REG1*⁺ homozygous strain at both 30 and 37°C. The strength of the suppression appeared to be somewhat weaker than the haploid strain, suggesting that the

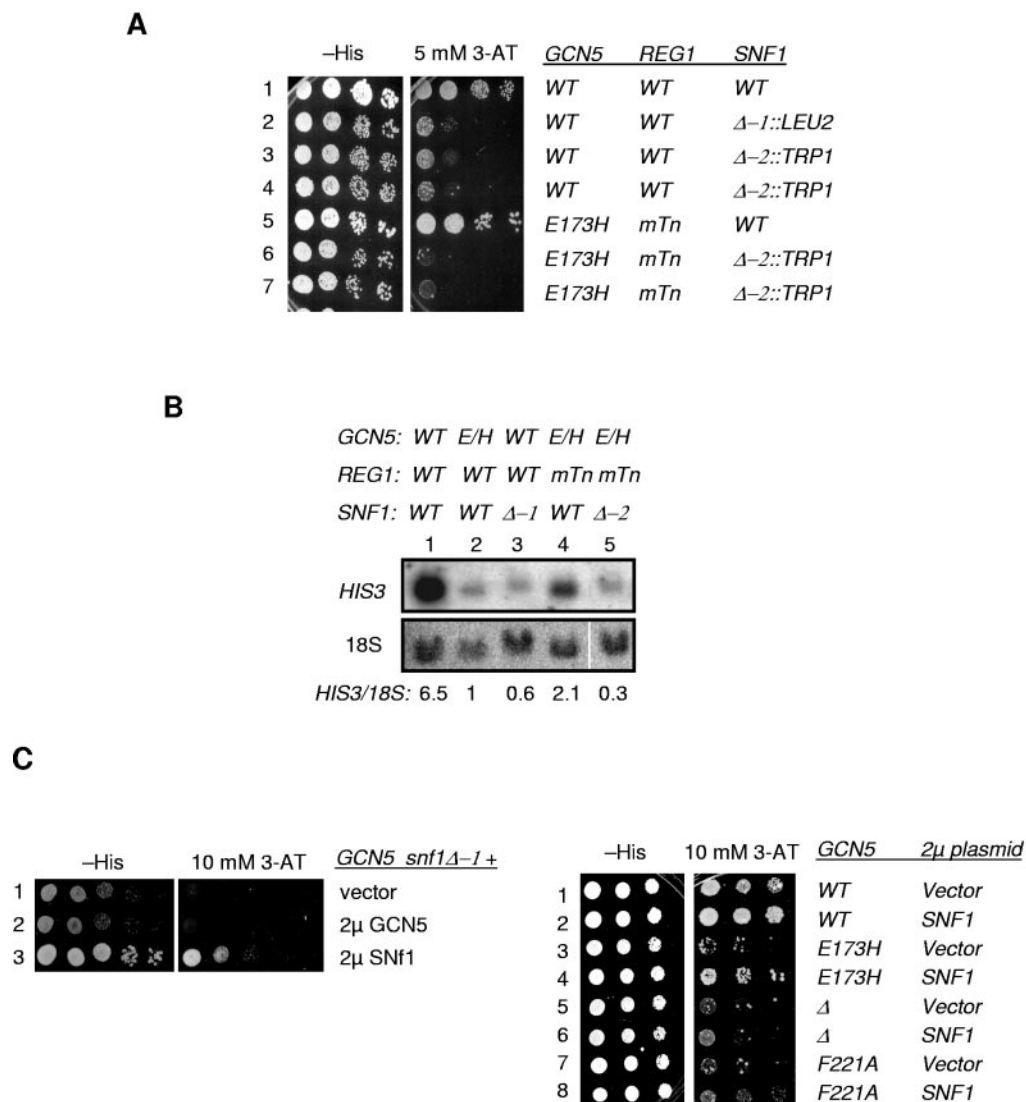


FIG. 4. *SNF1* is important for *HIS3* expression and *BGR* phenotypes. (A) Deleting *SNF1* causes hypersensitivity to 3-AT in *REG1* and *REG1::mTn* strains. The *snf1 $\Delta-2::TRP1$* allele contains a *TRP1* insertion at amino acid 109. The *snf1 $\Delta-1::LEU2$* allele lacks the entire ORF of *SNF1*. (B) Northern hybridization of some of the strains shown in panel A. Only induced transcription is shown. The ratios of *HIS3/18S* rRNA were normalized to the *gcn5* E173H sample (lane 2) and are shown at the bottom. (C) Overexpression of Snf1 protein also selectively rescues the E173H allele of *GCN5*. (Left panel) A multicopy *GCN5* construct does not rescue *snf1 $\Delta-1$* . (Right panel) *gcn5* E173H was selectively rescued by a multicopy *SNF1* construct. Growth at 37°C is shown.

Reg1(1-740) protein was a gain-of-function, semidominant suppressor.

Besides E173H, the F221A mutation also causes quantitative defects of Gcn5 functions in vitro and in vivo (Fig. 1B) (52). This mutation selectively impairs the ability of Gcn5 protein to bind acetyl coenzyme A (acetyl-CoA) (57, 76, 88), which is a prerequisite step for histone tail binding (88). It is thus likely that histone tails within the vicinity of the SAGA complex remain free from binding by the F221A mutant protein. In contrast, based on the studies of the E173Q mutant (89) and our yeast two-hybrid tests comparing different disabled *gcn5* mutants (M.-H. Kuo, unpublished data), the E173H allele most likely prolongs its association with both substrates because the catalytic process stalls after the ternary complex is formed. Because of the possible differential effects on histone

tail accessibility, we tested whether E173H, F221A, and a knockout allele of *GCN5* responded differently to Reg1(1-740) suppressor.

To test the allele specificity, the *gcn5 Δ* and *gcn5* F221A strains were engineered so that *REG1* was replaced with the *REG1(1-740)* allele, and the resultant strains were tested on 3-AT plates (Fig. 3B). Neither the F221A nor the complete knockout allele of *gcn5* was rescued by Reg1(1-740) (Fig. 3B, rows 6 and 8). The strong preference for the E173H allele suggests that Reg1 protein may interact directly with Gcn5 protein or may control Gcn5 at a step(s) subsequent to the formation of the Gcn5-acetyl-CoA-histone ternary complex.

We further tested whether Reg1(1-740) protein rescued other *gcn5 $^{-}$* phenotypes. Figure 3C shows that all three *gcn5 $^{-}$* mutants exhibited severe growth retardation in yeast extract-

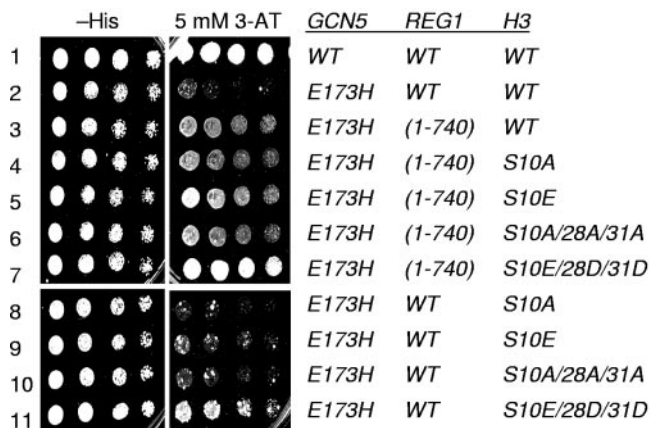


FIG. 5. H3 Ser10 phosphorylation is not required for the *BGR* phenotypes. Yeast strains expressing the desired H3 mutants were tested on 3-AT plates at 37°C. Strains derived from this background (2) were more sensitive to 3-AT. The choice of Asp or Glu in site-directed mutagenesis was based on whether a restriction site could be generated.

peptone-glycerol and yeast extract-peptone-ethanol media, as previously reported (58). Moreover, *gcn5*⁻ cells were also sensitive to 100 mM hydroxyurea, an inhibitor of DNA replication. However, neither the *mTn* allele nor the clean truncation of Reg1(1-740) protein was able to suppress any of these defects. The failure to suppress other phenotypes, such as caffeine sensitivity, and the inability to use galactose or sucrose were also observed (data not shown). In contrast, the sporulation defects (12) of a *gcn5* E173H homozygous strain were partially rescued (not shown). We conclude that the Reg1(1-740) protein suppresses only a subset of Gcn5 target genes.

Snf1 protein plays a critical role for *HIS3* expression. Reg1 protein is best known to inhibit the kinase activity of Snf1 protein and consequently prevents the expression of many genes when glucose is abundant (see the introduction), a function termed glucose repression. Snf1 protein derepresses the expression of these genes via several mechanisms, including histone H3 phosphorylation (59, 60). Phosphorylated H3 was shown to bind Gcn5 protein at a higher affinity (15, 17). It thus seems plausible that the hypoacetylation phenotype caused by *gcn5* mutations may be compensated for by hyperphosphorylation of H3, which either helps anchor Gcn5 protein to the *HIS3* promoter or by itself provides an environment suitable for stronger *HIS3* expression.

To examine the link between Gcn5, Snf1, and H3 phosphorylation, we first tested whether Snf1 protein was involved in *HIS3* expression. To this end, we created two deletion alleles of *SNF1*. The *snf1Δ-1::LEU2* mutant had the entire ORF replaced with a *LEU2* marker. However, this marker was incompatible with the *mTn-lacZ-LEU2* insertion mutant; we thus created another allele, *snf1Δ-2::TRP1*, that was truncated after amino acid 108. Figure 4 shows that both *snf1* mutations caused obvious growth defects on 3-AT plates (Fig. 4A, rows 2 to 4) as well as impaired *HIS3* expression (Fig. 4B, lane 3, and data not shown), demonstrating that the Snf1 protein was also a critical transcriptional regulator for *HIS3*. We next tested whether the Snf1 protein was important for the suppression. Deleting *SNF1* from the original *gcn5* E173H *REG1::mTn-lacZ* sup-

pressor strain significantly attenuated the suppression phenotypes (Fig. 4A, rows 6 and 7, and B, lane 5). Thus, Snf1 protein is critical for normal and Reg1(1-740)-mediated *HIS3* activation.

To see how Gcn5 and Snf1 proteins may genetically interact to activate *HIS3*, we examined whether overexpressing one of these two enzymes can rescue the *HIS3* expression defects caused by a mutation of the other. While a 2 μ m multicopy *GCN5* construct was unable to rescue the 3-AT hypersensitivity of the *snf1Δ-2::TRP1* strain (Fig. 4C, left panel), overproduction of Snf1 protein effectively rescued the E173H allele of *gcn5* (Fig. 4C, right panel, compare rows 3 and 4). On the other hand, overproduction of a catalytically inactive mutant of Snf1, K83R, failed to rescue the *gcn5*⁻ phenotypes (data not shown), suggesting that the kinase activity was essential for the suppression. Intriguingly, neither deletion nor the F221A allele of *gcn5* responded to the multicopy *SNF1* plasmid. Thus, the Snf1 multicopy suppressor displays an allele specificity similar to that of Reg1(1-740). Furthermore, in the presence of a functional *GCN5*, overproduction of Snf1 protein yielded higher resistance to 3-AT (Fig. 4C, right panel, row 2), very similar to the *GCN5*⁺ *REG1(1-740)* strain (Fig. 3B, row 2).

Taking together the above results, as well as the reports that Reg1 and Snf1 interact genetically and physically for transcription of several inducible genes (see the introduction), it seems likely that Snf1 may be part of the mechanism by which Reg1(1-740) protein suppresses the E173H mutant allele.

H3 Ser10 phosphorylation is not responsible for *bgr* suppression. To test whether H3 Ser10 phosphorylation contributes to the *BGR* phenotypes, we used a yeast strain in which both copies of each of the four core histone genes had been deleted (2). Viability of the cells was supported by a low-copy-number plasmid bearing wild-type histone genes and a *URA3* marker. The desired histone mutations can be introduced into an otherwise identical construct containing a *LEU2* nutrient marker. After transforming the latter plasmid that delivered the specific histone mutation(s), the wild-type histone genes were shuffled out by 5-FOA selection, leaving the mutant allele as the sole copy for histone expression. Additionally, *GCN5* and *REG1* were replaced with the E173H and *REG1(1-740)* alleles, respectively. 3-AT resistance was then compared among different *LEU*⁺ *Ura*⁻ strains as shown in Fig. 5. In this genetic background, Reg1(1-740) also effectively rescued the E173H mutant. However, the S10A mutation did not impose a discernible effect on cellular growth (Fig. 5, compare rows 3 and 4), ruling out a critical role played by phosphorylated Ser10 alone. Within the amino-terminal tail domain of histone H3, Ser28 and Ser31 share sequence similarity with Ser10 (⁷ARKSTGG and ²⁵ARKSAPSTGG). Although Snf1 protein has not been shown to phosphorylate either serine residue, Ser28 can be phosphorylated by the Aurora family kinases for chromatin condensation during mitosis (16, 31, 82). We were curious about the possibility that the Snf1 kinase activity might “spill over” to these two residues in the *REG1(1-740)* strain. Thus, a triple Ser-to-Ala mutant, S10A/S28A/S31A, was introduced to the *gcn5* E173H *REG1(1-740)* background. These cells still exhibited robust growth in the presence of 3-AT (Fig. 5, row 6), further supporting the notion that H3 phosphorylation was unlikely to be the driving force for the observed *BGR* phenotypes. Consistent with this, neither single nor triple Ser-to-Ala mutations exacerbated the 3-AT hypersensitivity caused

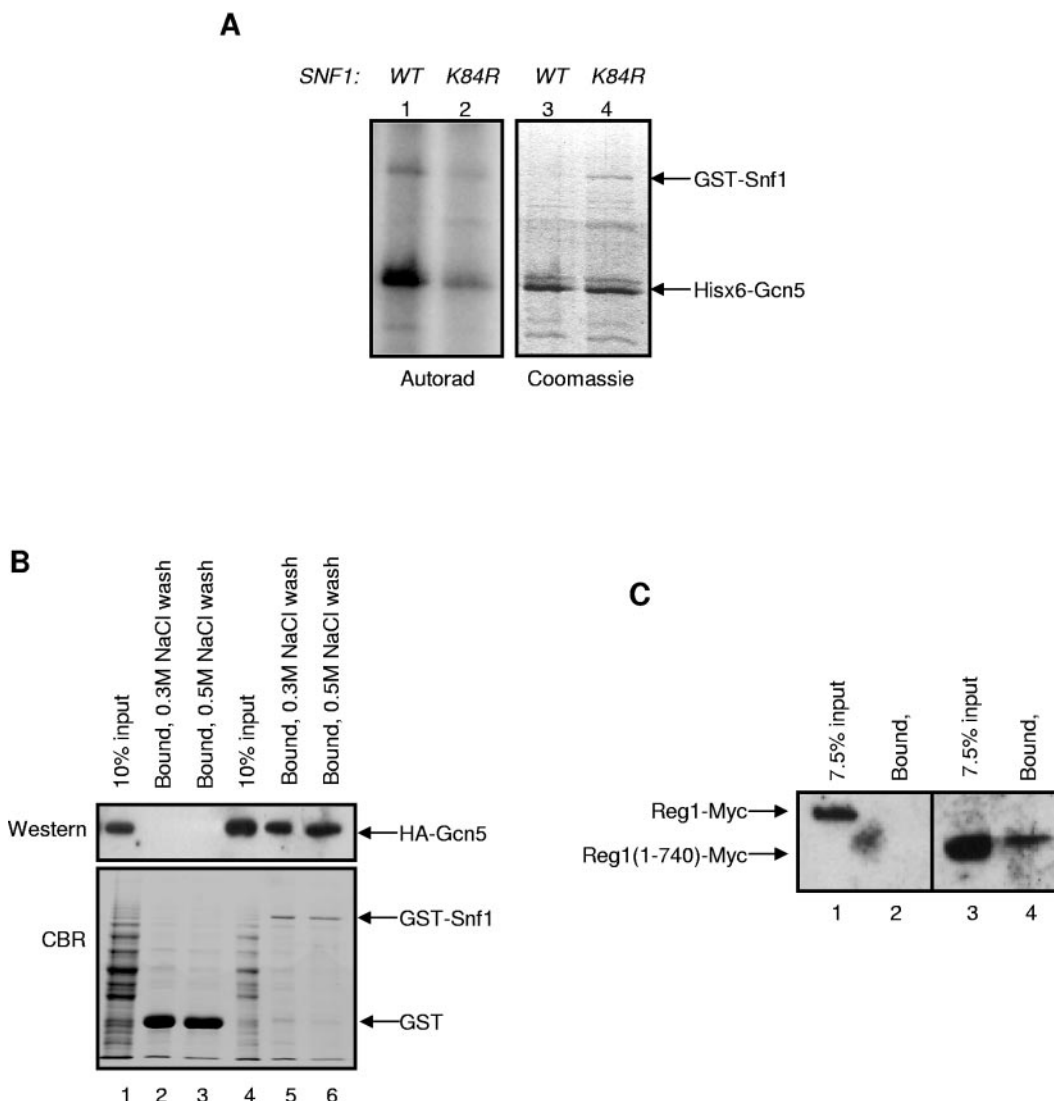


FIG. 6. Biochemical interactions of Gcn5/Snf1 and Gcn5/Reg1(1-740) proteins. (A) Recombinant Gcn5 is phosphorylated by the wild-type but not the K84R Snf1 protein. GST-Snf1 or GST alone was purified from yeast and incubated in the presence of [γ - 32 P]ATP with recombinant Gcn5 protein. (B) Copurification of Gcn5 and Snf1 proteins from yeast. HA-Gcn5 and GST-Sfn1 or GST alone were expressed in yeast, and the whole-cell extracts were subjected to glutathione affinity purification. The bound materials were washed with 0.3 or 0.5 mM NaCl prior to SDS-PAGE and Western analyses using an anti-HA antibody (top). (C) Copurification of Reg1(1-740) and Gcn5 proteins from yeast. Gcn5 was C-terminally tagged with protein A and coexpressed in yeast strains with Reg1-Myc or Reg1(1-740)-Myc recombinant proteins. Whole-cell lysates were bound to immunoglobulin G beads and analyzed by SDS-PAGE and anti-Myc Western analyses.

by the E173H mutation in a *REG1*⁺ background (Fig. 5, compare rows 2, 8, and 10). We therefore conclude that Ser10 phosphorylation, though important for activation of several other genes, does not contribute appreciably to Gcn5 and Snf1 protein-mediated *HIS3* expression. Thus, Snf1 protein most likely controls *HIS3* expression by a novel, H3 phosphorylation-independent mechanism(s).

While preventing H3 phosphorylation imposes no apparent effect on the Reg1(1-740) protein-generated suppression, we were nonetheless interested in knowing whether a constitutively phosphorylated H3 would be sufficient to bring about a chromatin environment that suppresses the *gcn5* E173H transcriptional defects. Toward this end, Ser10, Ser28, and Ser31 were replaced by aspartate or glutamate that mimicked the

negatively charged phosphorylation state. Cellular growth in the presence of 3-AT was then assessed. While a single S10E mutation yielded very few differences in *REG1* or *REG1* (1-740) background (Fig. 5, rows 5 and 9), the triple acidic mutation clearly brought about stronger resistance to 3-AT (Fig. 5, rows 7 and 11). Since this phenotype was independent of the *REG1* status, we conclude that constitutive negative charges at the amino terminus of H3 represent another bypass of Gcn5 requirement suppressor.

Physical interactions of Gcn5, Snf1, and Reg1(1-740). The above data place both Reg1 and Snf1 proteins to the regulatory circuitry of *HIS3* and likely other amino acid starvation-inducible genes. The ability of Reg1(1-740) protein and overproduced Snf1 kinase to rescue preferentially the E173H mutant

suggests an intriguing possibility that Gcn5 protein is a functional target for the Snf1 kinase. To test this hypothesis, we purified a wild-type and a catalytically inactive (K84R) GST-Snf1 protein from yeast (35) and incubated these two preps with recombinant Gcn5 protein expressed in *E. coli*. [γ - 32 P]ATP was included in the reactions to track the phosphorylation status of Gcn5. Figure 6A shows that Gcn5 protein was indeed phosphorylated in the presence of the wild-type Snf1 protein. The K84R mutation effectively diminished Gcn5 phosphorylation, indicating that Snf1 protein was responsible for Gcn5 protein phosphorylation.

Intrigued by the *in vitro* phosphorylation results, we further tested whether Gcn5 and Snf1 proteins interacted *in vivo*. To this end, we epitope tagged Gcn5 with HA at its amino terminus. Two yeast strains expressing GST-Snf1 or GST were transformed with the HA-GCN5 construct and subjected to one-step purification with a glutathione matrix. After extensive washing, the bound materials were resolved by SDS-PAGE and probed with an anti-HA antibody. Figure 6B shows apparent copurification of the HA-Gcn5 protein with GST-Snf1 but not GST alone. Literally identical results were obtained in reciprocal experiments (i.e., immunoprecipitation with the anti-HA antibody, followed by Western analyses to quantify Snf1 protein in the precipitate) (not shown), confirming the *in vivo* association between Gcn5 and Snf1 proteins.

We then asked whether Reg1 protein also associated with Gcn5 protein. Figure 6C shows that a Myc-tagged Reg1(1-740) protein was also present in the crude preparation of an epitope-tagged Gcn5 protein. Intriguingly, the full-length Reg1-Myc protein was not detected under the same condition (Fig. 6C, first two lanes), consistent with the gain-of-function trait of the Reg1(1-740) suppressor protein.

DISCUSSION

A putative noncatalytic function of Gcn5 protein. The histone acetyltransferase activity of Gcn5 protein is critical for the expression of multiple yeast genes. Point mutations that eliminate the HAT activity of Gcn5 protein cause defects in promoter acetylation and in transcriptional activation of such model genes as *HIS3* and *PHO5* (7, 52, 73, 100). While these results provide solid evidence that Gcn5 protein uses its HAT activity to activate transcription, microarray studies also showed that a *gcn5* knockout strain has transcriptional defects in more genes than does a strain expressing a catalytically inactive mutant (43), suggesting that Gcn5 protein may perform noncatalytic roles in gene expression. Indeed, Jacobson and Pillus showed that a catalytically inactive Gcn5 protein counteracts transcriptional silencing at subtelomeric loci (46). Such noncatalytic functions of Gcn5 protein may be unveiled by characterizing point mutations that abrogate the catalytic power of Gcn5 protein but permit other functions to be exerted. This notion seems to be consistent with the data presented in this work. For example, *HIS3* and *HIS4* expression are effectively rescued by the Reg1(1-740) suppressor (Fig. 1D and 2C) in the E173H but not the knockout background. No restoration of histone H3 acetylation was detected, suggesting one possibility that the noncatalytic function of the E173H allele is selectively enhanced by Reg1(1-740) protein. This function is likely synergistic with its catalytic counterpart, as

more pronounced resistance to 3-AT is exhibited by *GCN5*⁺ *REG1*(1-740) and *GCN5*⁺ multicopy *SNF1* strains (Fig. 3B and 4C).

It is also intriguing that the F221A allele is refractory to Reg1(1-740) and higher doses of Snf1 protein. Several other suppressors that are currently characterized by us do not show such unique allele specificity (Y. Liu, X. Xu, and M.-H. Kuo, unpublished data). Molecularly, E173H and F221A mutations abrogate the HAT activity of Gcn5 via different mechanisms and may have different impacts on histone tails. F221A impairs acetyl-CoA binding (57, 88, 93), whereas E173H blocks the nucleophilic attack on the bound acetyl-CoA (89). Association of acetyl-CoA is prerequisite to histone tail binding (88, 89). After the transfer of the acetyl group to histone within the ternary complex, the acetylated histone dissociates first and then follows the consumed coenzyme A. Thus, blocking the association between Gcn5 and acetyl-CoA by the F221A mutation likely prevents Gcn5 protein from binding to the substrate histone, rendering the latter susceptible to other unregulated or untimely chromatin binding and modulating activities. The E173H mutation, on the other hand, may lock Gcn5, acetyl-CoA, and the histone tail in a ternary complex, thus preventing possible usage or modifications of the histone tail by other activities. In addition, it remains a strong possibility that Gcn5 protein uses nonhistone protein substrates (68). If so, the retention of one of these proteins by the E173H mutant enzyme may exacerbate the histone hypoacetylation defects.

Furthermore, only a subset of defects associated with *gcn5*⁻ mutants can be rescued by Reg1(1-740) (Fig. 3C). Together, it is highly likely that Gcn5 uses multiple mechanisms to activate transcription in a target gene (or transcriptional activator)-dependent manner.

Reg1(1-740) protein is a gain-of-function suppressor. Reg1 protein is a regulatory subunit for Glc7, an essential and multifunctional type I protein phosphatase (95). Reg1 protein also interacts with several other proteins, including Snf1 (61, 79) and the yeast 14-3-3 homologues, Bmh1 and Bmh2 proteins (22). The binding domains for these proteins are all within the first 500 amino acids that are conserved among Reg1 protein homologues (22, 23, 61). These domain are preserved in our *REG1*(1-740) suppressor allele, suggesting that the prototypical functions of Reg1 protein are not impaired by the C-terminal truncation.

The Reg1(1-740) protein lacks about one third of the total length. The truncation occurs immediately before a stretch of acidic residues (15 of 19 residues are Asp or Glu), and the deleted portion is rich in serine, threonine, and acidic residues (16% Ser, 4.4% Thr, 8.8% Asp, and 7.3% Glu). Little is known about the molecular functions or potential partners of this part of the Reg1 protein. Preliminary sequence search reveals no clear homologues to this region across species (data not shown). Contrary to the gain-of-function *BGR* phenotypes, this C-terminal region is dispensable for glucose repression. For example, Dombek et al. showed that the C-terminal deletion of Reg1 protein (up to residue 693) does not cause appreciable derepression of *ADH2* or *SUC2* (23). Shirra and Arndt reported that a Reg1 protein missing the last 80 amino acids is able to fully complement a recessive *reg1-326* mutant (83). Indeed, we have no evidence of transcriptional derepression of those glucose-repressible genes in the *REG1*(1-740) back-

ground (Y. Liu and M.-H. Kuo, unpublished). It is possible that the carboxyl-terminal third of Reg1 protein interacts with a negative regulator(s), or another region of Reg1 protein in *cis*, that restricts specifically the *HIS3* expression-related functions of Reg1 protein. Perhaps this negative regulator selectively controls the residual non-HAT function of the E173H mutant of Gcn5 protein. Upon deleting this Ser-Thr-Asp-Glu-rich domain, the negative effect of this regulator diminishes, hence unleashing the non-HAT function of Gcn5 protein for *HIS3* activation. This view is consistent with the affinity purification data (Fig. 6) that the Reg1(1-740) but not the full-length Reg1 protein can be copurified with an epitope-tagged Gcn5 protein.

It is important that the suppressing power of Reg1(1-740) protein is abrogated by deleting *SNF1*. While this result alone does not prove that Snf1 protein acts downstream of the Reg1(1-740) suppressor, considering the well-established interaction between Reg1 and Snf1 proteins, we suggest that at least part of the suppressor function of Reg1(1-740) protein is mediated through Snf1 protein. However, we cannot rule out the existence of an intermediary step(s)/factor(s) for the suppression.

One probable factor involved in the *BGR* phenotype is the type 1 protein phosphatase Glc7. Reg1 is one of several regulators of the essential Glc7 enzyme. Unfortunately, our attempts to link Glc7 protein to the *BGR* phenotypes failed to generate conclusive data. Using several known *glc7* point mutations that cause phenotypes in glycogen metabolism and/or glucose repression, we indeed found a few able to confer strong resistance to 3-AT in the absence of a functional Gcn5 protein. However, such elevated 3-AT resistance was not accompanied by increased *HIS3* transcription (Y. Liu and M.-H. Kuo, unpublished). This disparity probably arises from the fact that Glc7 protein controls multiple cytoplasmic and nuclear functions (e.g., see references 87 and 101). Changes in the metabolism and flux of 3-AT may render yeast cells resistant to 3-AT with a low level of *HIS3* transcription. The possible involvement of *GLC7* in *HIS3* regulation awaits further investigation when more mutant *glc7*⁻ alleles are available.

Reg1 protein was recently shown to be purified in a complex containing two yeast 14-3-3 homologues, Bmh1 and Bmh2 proteins, and heat shock proteins Ssd1 and Ssd2 (22). Deleting *BMH1* or *BMH2* did not appreciably alter the ability of Reg1(1-740) protein to rescue the *gcn5* E173H mutant (X. Xu and M.-H. Kuo, data not shown), indicating that these two proteins are not part of the suppression mechanism. Alternatively, functional redundancy between Bmh1 and Bmh2 proteins (92% identical) (98) may account for the lack of phenotypes in *bmh1*Δ and *bmh2*Δ strains.

Interestingly, the gain-of-function nature of the Reg1(1-740) suppressor, as well as the phenotypic similarity between Reg1(1-740) and overexpressed Snf1 protein, are at odds with the well-characterized antagonistic relationship with Snf1 protein (see the Introduction). We suggest that the functional relationship between these two proteins may be gene dependent. One precedent for this type of functional variation was reported for Spt3/8 proteins on TBP recruitment. Spt3 protein genetically and physically interacts with TBP (25). While Spt3 protein is required for TBP binding to the TATA elements of

GAL1 and *ADH2* (8, 9, 24, 55), it also plays a negative role in TBP-TATA interaction in other cases (7, 105).

Snf1 protein activates *HIS3* in an H3 phosphorylation-independent mechanism. Snf1 protein is a member of the AMP-activated protein kinase family that serves as a metabolic sensor in eukaryotic cells (37). It thus seems reasonable that Snf1 protein also contributes to the regulation of amino acid biosynthesis genes as shown in this work. Despite the functional interaction between Gcn5 and Snf1 proteins for *INO1* activation (15, 17, 58–60), the H3 phosphorylation function of Snf1 protein is unlikely to be a major determinant in *HIS3* expression (Fig. 5). However, we cannot rule out the possibility of phosphorylation at other residues or histones by Snf1 protein. In addition, genetic data showed that Srb/mediator complex and TBP are also potential substrates of Snf1 kinase (49, 83).

It is interesting that Snf1 protein can modify a recombinant Gcn5 protein and that these two proteins are copurified from yeast (Fig. 6). We do not yet know the site(s) modified by Snf1 protein in vitro, nor has it been tested whether Gcn5 protein is phosphorylated in vivo. The human Gcn5 protein was shown to be modified and inhibited by the DNA-dependent kinase (6). In our hands, the in vitro-phosphorylated Gcn5 also seems to exhibit a slightly lower activity on histones H3 and H4 (X. Xu and M.-H. Kuo, unpublished). However, it remains an open question as to whether a phosphorylated Gcn5 protein behaves differently within the context of native complexes.

In conclusion, combining the data presented here and those reported by others, we propose a simple model that that Reg1(1-740) protein uses its newly adopted affinity for Gcn5, while maintaining the Snf1 interaction domain (79), to mediate the interaction between Gcn5 and Snf1 proteins. When Snf1 protein is brought to the vicinity of Gcn5, phosphorylation of Gcn5 protein or another factor(s) within or near the SAGA complex may provide the noncatalytic function that rescues the E173H mutation for effective activation of a subset of Gcn5 target genes.

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