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Nrg1 and Nrg2 Transcriptional Repressors Are Differently Regulated in Response to Carbon Source

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The Nrg1 and Nrg2 repressors of *Saccharomyces cerevisiae* have highly similar zinc fingers and closely related functions in the regulation of glucose-repressed genes. We show that *NRG1* and *NRG2* are differently regulated in response to carbon source at both the RNA and protein levels. Expression of *NRG1* RNA is glucose repressed, whereas *NRG2* RNA levels are nearly constant. Nrg1 protein levels are elevated in response to glucose limitation or growth in nonfermentable carbon sources, whereas Nrg2 levels are diminished. Chromatin immunoprecipitation assays showed that Nrg1 and Nrg2 bind DNA both in the presence and absence of glucose. In mutant cells lacking the corepressor Ssn6(Cyc8)-Tup1, promoter-bound Nrg1, but not Nrg2, functions as an activator in a reporter assay, providing evidence that the two Nrg proteins have distinct properties. We suggest that the differences in expression and function of these two repressors, in combination with their similar DNA-binding domains, contribute to the complex regulation of the large set of glucose-repressed genes.

The Nrg1 and Nrg2 proteins of *S. cerevisiae* are similar C_2H_2 zinc finger proteins that function as transcriptional repressors. Considerable evidence indicates that these two proteins have broad roles in regulation of glucose-repressed genes. Nrg1 was first identified by its role in glucose repression of the *STA1* (glucoamylase) gene (22). Nrg2 was identified by its two-hybrid interaction with Snf1 protein kinase, a component of a major glucose signaling pathway, and both Nrg proteins were shown to interact physically with Snf1 (30). The Nrg proteins contribute to repression of multiple glucose-repressed genes, including the *DOG2*, *SUC2*, *GAL*, *STA2*, and *FLO11* genes (10, 30, 33). Nrg1 and Nrg2 also function as negative regulators of haploid invasive growth and initiation of biofilm formation, which are cellular responses to glucose limitation (3, 10, 25).

The Nrg repressors have also been implicated in response to other environmental conditions. Both are negative regulators of diploid pseudohyphal growth (10), which occurs in response to nitrogen limitation; this function may be related to that of the Nrg1 ortholog of *Candida albicans*, which represses filamentous growth and expression of hypha-specific genes (2, 20). *NRG1* is repressed by the alkaline pH response regulator Rim101, and Nrg1 represses alkaline pH-induced genes (11). Finally, levels of *NRG2* RNA are induced by zinc limitation (18) and alkaline pH (12).

Previous evidence indicated that Nrg1 and Nrg2 have closely related functions with respect to glucose regulation (10, 29, 30), consistent with the strong similarity of their DNA-binding domains (84% identity). However, we considered the possibility that these two proteins have distinct functions in glucose repression for two reasons. First, the Nrg proteins are less similar outside their DNA-binding domains (27% identity) and, hence, may interact differently with regulatory proteins or other transcription factors. Second, *NRG1* and *NRG2* RNAs are regulated differently during the diauxic shift. *NRG1* RNA levels are induced 2.7-fold, whereas expression of *NRG2* does not change (4). However, these results are not easily reconciled with a report that *NRG1* RNA levels were sixfold lower during growth in glycerol-ethanol than in glucose (22).

We have here explored the differences between *NRG1* and *NRG2* with respect to regulation of their expression in response to carbon source at the levels of both RNA and protein. We have used chromatin immunoprecipitation assays to assess the DNA binding of Nrg1 and Nrg2 and its regulation by glucose signals. Finally, we used a reporter assay to provide evidence that the two proteins have different properties. The findings support the view that Nrg1 and Nrg2 are distinct with respect to both regulation of expression and function.

MATERIALS AND METHODS

Strains and genetic methods. *S. cerevisiae* strains used in this study are listed in Table 1. To create hemagglutinin (HA)-tagged *NRG* genes at the genomic loci, we amplified the region of pCDB5 containing the triple HA coding sequence, the *ADH1* terminator, and *kanMX6* by PCR with primers containing homology to the desired site of genomic integration. The PCR fragment was used to transform MCY4702, and recombinants were selected by kanamycin resistance and confirmed by PCR. The *gal83*Δ allele has been described previously (29). Rich medium was yeast extract-peptone (YEP) containing 2% glucose or the indicated carbon source, and synthetic complete (SC) medium lacked appropriate supplements to select for plasmids (26).

Plasmids. Plasmid pCDB5 is a derivative of pFA6A-GFP(S65T)-kanMX6 (15) in which the green fluorescent protein sequence was replaced by a triple HA sequence by homologous recombination in yeast. To construct pV43 and pV44, which express LexA-Nrg2 and LexA-Nrg1, respectively, PCR fragments containing the open reading frames flanked by BamHI sites were cloned into vector pEG202.

Preparation of RNA and Northern blot analysis. Cells (50 ml) were grown to an optical density at 600 nm of 1 in YEP containing 2% glucose and shifted to YEP containing 0.05% glucose for 1 h or were grown in YEP containing 2% glycerol plus 2% ethanol. Cells were collected by filtration, resuspended in 0.7 ml of TES (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]), and frozen in liquid nitrogen. An equal volume of acid phenol was added to the sample, and cells were incubated at 65°C for 1.5 h with vortexing 10 times for 30 s at 3-min intervals and then for 30 s every 10 min. Samples were extracted four times with an equal volume of phenol and then twice with chloroform. RNA

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TABLE 1. Strains used in this study[a]

Strain	Genotype	Reference
MCY829	*MATα his3Δ200 lys2-801 ura3-52*	27
MCY1974	*MATα ssn6Δ9 ade2-101 his3Δ200 lys2-801 trp1Δ1 ura3-52*	27
MCY4661	*MAT***a** *nrgΔ1::kanMX6 nrgΔ2::natMX4 his3Δ leu2Δ ura3Δ*	This study
MCY4702	*MAT***a** *his3Δ leu2Δ ura3Δ*	29
MCY4744	*MAT***a** *NRG1-HA::kanMX6 his3Δ leu2Δ ura3Δ*	This study
MCY4746	*MAT***a** *gal83Δ his3Δ leu2Δ ura3Δ*	This study
MCY4748	*MAT***a** *NRG1-HA::kanMX6 gal83Δ his3Δ leu2Δ ura3Δ*	This study
MCY4751	*MAT***a** *NRG2-HA::kanMX6 his3Δ leu2Δ ura3Δ*	This study
MCY4753	*MAT***a** *NRG2-HA::kanMX6 gal83Δ his3Δ leu2Δ ura3Δ*	This study
MCY4758	*MAT***a** *snf1Δ10 his3Δ leu2Δ ura3Δ*	This study
MCY4760	*MAT***a** *NRG1-HA::kanMX6 snf1Δ10 his 3Δ leu2Δ ura3Δ*	This study
MCY4768	*MAT***a** *NRG2-HA::kanMX6 snf1Δ10 his3Δ leu2Δ ura3Δ*	This study
MCY4778	*MAT***a** *NRG1-HA::kanMX6 sip1Δ::kanMX6 his3Δ leu2Δ ura3Δ*	This study
MCY4798	*MAT***a** *NRG1-HA::kanMX6 sip2Δ::LEU2 his3Δ leu2Δ ura3Δ*	This study
MCY4802	*MAT***a** *NRG1-HA::kanMX6 gal83Δ sip2Δ::LEU2 his3Δ leu2Δ ura3Δ*	This study

[a] All strains have the Σ1278b genetic background except MCY829 and MCY1974, which have the S288C background.

was precipitated with ethanol and resuspended in water. RNAs (20 μg) were separated by electrophoresis on a 1.2% agarose–morpholinepropanesulfonic acid (MOPS) gel containing formaldehyde and transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech). Probes were ^{32}P-labeled with Ready-To-Go DNA labeling beads (Amersham Biosciences). Levels of RNA were determined relative to levels of the small nucleolar RNA U3 encoded by *SNR17A* and *SNR17B*.

Western analysis. Cells (50 ml) were collected and resuspended in 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM dithiothreitol, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor cocktail (Roche Molecular Biochemical). Acid-washed glass beads were added, and cells were lysed in a Biospec mini-bead beater on the highest setting for 3 min in 1-min intervals separated by 2 min on ice. Extracts were frozen overnight. Proteins (80 to 100 μg) were separated by SDS-polyacrylamide gel electrophoresis in 12% acrylamide. The gel was analyzed by immunoblotting with monoclonal anti-HA (Babco HA.11 or 12CA5). Antibodies were detected by chemiluminescence with ECLPlus (Amersham Biosciences).

Chromatin immunoprecipitation. Cells were grown to mid-log phase in rich medium containing the specified carbon source. Chromatin immunoprecipitation was carried out as described previously (1) with anti-HA antibody (F7; Santa Cruz Biotechnology) and protein A-Sepharose beads (Amersham Pharmacia). To analyze the recovered DNA, the following oligonucleotide pairs were used for PCR: *RPI1*, 5′-GACGGGTAATCCTGTTAGTG plus 5′-CGGGTGTGTTGTA AGAATGG and 5′-CGTTGACTAATTTCGGAGTC plus 5′-GGTTACCTTC AAGCAAATCC; CYC7, 5′-GGTATACGAGCTAGCAGGAC plus 5′-CCCTT GCGGCCACTTATAGT; *FLO11*, 5′-CATCTGTGTGCCATGTCAGA plus 5′-GGTGTATGGCTCGGCTCTCG; *POL1*, 5′-TGCACCAGTTAATTCTAAAA AGGCA plus 5′-AAACACCCTGATCCACCTCTGAA. PCR was performed with 15-μl reaction mixtures with Hotstar *Taq* polymerase (Qiagen), an annealing temperature of 56°C, and a 30-s extension step for 29 cycles. PCR products were visualized on a 2.5% agarose gel. Quantitative PCR analysis was performed in real time with an Applied Biosystems 7700 sequence detector and with the *POL1* coding sequence as the control. Immunoprecipitation efficiency was calculated in triplicate by dividing the amount of PCR product from the immunoprecipitated sample by the amount of PCR product in the input sample. Data are presented as immunoprecipitation efficiencies over the *POL1* control (*n*-fold).

β-Galactosidase assays. Cells were grown in selective SC to an optical density at 600 nm of 0.5, collected, and frozen at −20°C. β-Galactosidase activity was assayed in permeabilized cells (7) and expressed in Miller units (19).

RESULTS

***NRG1* and *NRG2* RNA levels are differently regulated in response to glucose.** To further understand the roles of Nrg1 and Nrg2 in glucose repression, we first assessed the expression of the cognate RNAs in wild-type cells grown in different carbon sources. Cells of the Σ1278b genetic background were grown to mid-log phase in 2% glucose and were shifted to 0.05% glucose for 1 h. Northern blot analysis showed that expression of *NRG1* RNA increased in response to this acute glucose starvation, whereas *NRG2* RNA levels remained nearly constant, with a small decrease (Fig. 1). To examine RNA levels in cells grown for an extended period in the absence of glucose, we grew cells in 2% glycerol–2% ethanol. *NRG1* RNA levels were much higher than in glucose-grown cells, whereas *NRG2* RNA levels were the same. Thus, the *NRG1* and *NRG2* genes are differently regulated in response to carbon source.

Snf1 protein kinase, a key component of a signaling pathway that is inhibited by glucose, has broad roles in carbon source-responsive transcriptional control (8). Analysis of *snf1*Δ mutant cells showed that *NRG1* and *NRG2* are differently regulated by Snf1 protein kinase (Fig. 1). *NRG1* RNA levels did not increase in response to glucose limitation in *snf1*Δ mutant cells,

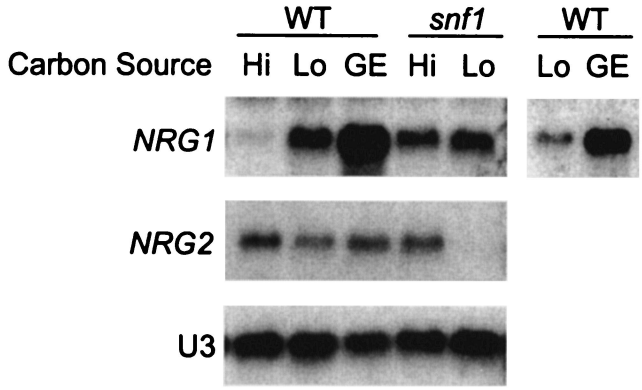

FIG. 1. Regulation of *NRG1* and *NRG2* RNA levels in response to carbon source. Cultures of wild-type (WT) and *snf1*Δ strains (MCY4702 and MCY4758) were grown in YEP containing high (2%) glucose (Hi) and were shifted to low (0.05%) glucose (Lo) for 1 h. Wild-type cells were also grown in 2% glycerol–2% ethanol (GE); *snf1*Δ cells do not grow in a nonfermentable carbon source. RNA was prepared and subjected to Northern blot analysis. Blots were hybridized with probes specific for *NRG1*, *NRG2*, and the loading control, U3 RNA. Autoradiograms are shown. The panel at the right shows a shorter exposure for two lanes.

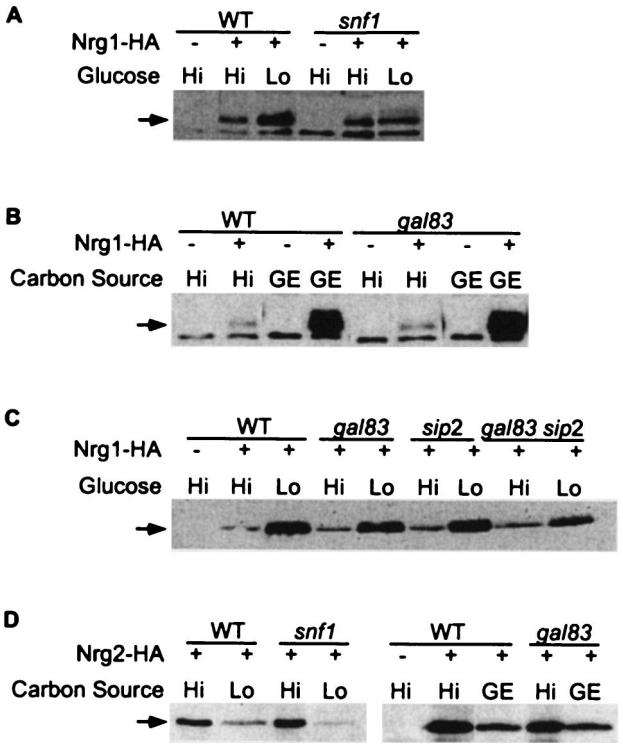

FIG. 2. Regulation of Nrg1 and Nrg2 protein levels in response to carbon source. Wild-type (WT) and mutant strains of the indicated genotype (Table 1) expressed Nrg1-HA or Nrg2-HA from the chromosomal locus (+) or expressed the wild-type untagged proteins (−). Cultures were grown in YEP containing high (2%) glucose (Hi) and were shifted to low (0.05%) glucose (Lo) for 1 h or were grown in 2% glycerol–2% ethanol (GE). For the experiments shown in panels C and D, the shift to low glucose was for 3 h. Proteins were resolved by SDS–12% polyacrylamide gel electrophoresis, and proteins were blotted and detected with anti-HA antibody 12CA5 (A and B) or Babco HA.11 (C and D). Panel D shows two blots from different experiments. Arrows indicate the Nrg-HA proteins.

but levels were higher than in wild-type cells during growth in glucose. In contrast, *NRG2* RNA was nearly undetectable in the mutant cells after a shift to glucose-limiting conditions, but the RNA levels were the same in the *snf1*Δ mutant and the wild type during growth in glucose. The *snf1*Δ mutant does not grow in glycerol-ethanol.

Nrg1 and Nrg2 protein levels are differently regulated in response to glucose. To determine whether further regulation occurs at the protein level, we examined the expression of the proteins in cells grown in different carbon sources by immunoblot analysis. C-terminally HA-tagged Nrg1 (Nrg1-HA) and Nrg2 (Nrg2-HA) were expressed from their native promoters at the chromosomal loci. Both proteins were functional, as assayed by their ability to confer glucose-regulated expression to a *STA2-lacZ* reporter (data not shown); this reporter is sensitive to deletion of either gene (10). When wild-type cells growing exponentially in 2% glucose were shifted to 0.05% glucose for 1 h, Nrg1-HA levels increased (Fig. 2A), and levels were much higher during steady-state growth in 2% glycerol–2% ethanol (Fig. 2B). In contrast, Nrg2-HA protein levels decreased when cells were shifted from high-glucose to glucose-starvation conditions and were lower during growth in glycerol-ethanol than during growth in glucose (Fig. 2D). Thus, the levels of these two repressors are differently regulated in response to carbon source.

Comparison of the immunoblots and Northern blots (Fig. 1 and 2) indicates that in wild-type cells, the changes in Nrg1 protein levels parallel those observed for *NRG1* RNA. In contrast, Nrg2 protein levels decreased in response to glucose deprivation, whereas RNA levels remained nearly the same, suggesting that the regulation of Nrg2 protein levels involves posttranscriptional control.

Previous evidence for physical and functional interaction of Snf1 kinase with the Nrg proteins raised the possibility that Snf1 modulates Nrg protein levels. Immunoblot analysis of *snf1*Δ mutant cells during growth in high glucose and after a shift to low glucose showed that Nrg1-HA protein levels remained the same (Fig. 2A), as did the RNA levels (Fig. 1). However, the protein levels were comparable to those of glucose-grown wild-type cells, whereas the RNA levels in the *snf1*Δ mutant were considerably higher, suggesting that Snf1 positively regulates Nrg1 at the protein level. Nrg2-HA levels in the *snf1*Δ mutant reflected the RNA levels; however, because there is little or no RNA in the mutant after a shift to low glucose, this experiment does not address whether Snf1 affects Nrg2 protein levels in glucose-limiting conditions.

Snf1 kinase exists in three forms containing alternate isoforms of the β subunit, Sip1, Sip2, or Gal83 (32). Genetic studies implicated the Snf1-Gal83 form of the kinase in antagonizing Nrg repressor function (29). Immunoblot analysis of *gal83*Δ, *sip1*Δ, and *sip2*Δ mutants showed that no single β subunit is required for control of Nrg1 protein levels (Fig. 2B and C and data not shown) and that the Snf1-Gal83 form of the kinase is not required for control of Nrg2 levels (Fig. 2D).

Nrg1 binds to DNA in the absence of glucose. These findings indicate that Nrg1-mediated repression of glucose-repressed genes is not regulated by controlling levels of Nrg1 protein, because levels are elevated under derepressing conditions, and previous studies showed that the nuclear localization of Nrg1 is not regulated by glucose (30). Another possible mechanism for regulating Nrg repressor function in response to glucose signals is through regulation of its binding to promoters. To determine whether the binding of Nrg1 to DNA is regulated in vivo, we assayed its binding to three different promoters by chromatin immunoprecipitation. Cells expressing Nrg1-HA were grown in high glucose and shifted to low glucose or were grown in glycerol-ethanol. Chromatin was immunoprecipitated with anti-HA, and the recovered DNA was amplified by PCR with primers specific for each promoter. Primers to the *POL1* coding sequence provided an internal control.

The *RPI1* promoter was chosen because Nrg1 binds directly to this promoter (14), and Northern blotting confirmed that Nrg1 negatively regulates *RPI1* gene expression in glucose-grown cells (data not shown). The immunoprecipitated DNA was amplified by PCR with primers for two regions encompassing CCCCT sequences. The *C. albicans* Nrg1 ortholog binds CCCCT (20), and an Nrg1 binding site in the *STA1* promoter includes CCCCT (22). In glucose-grown cells, Nrg1-HA bound to both regions of the *RPI1* promoter (Fig. 3A). Binding increased in response to glucose deprivation and was also higher during growth in glycerol-ethanol; Nrg1 protein levels also increased under these conditions (Fig. 2), al-

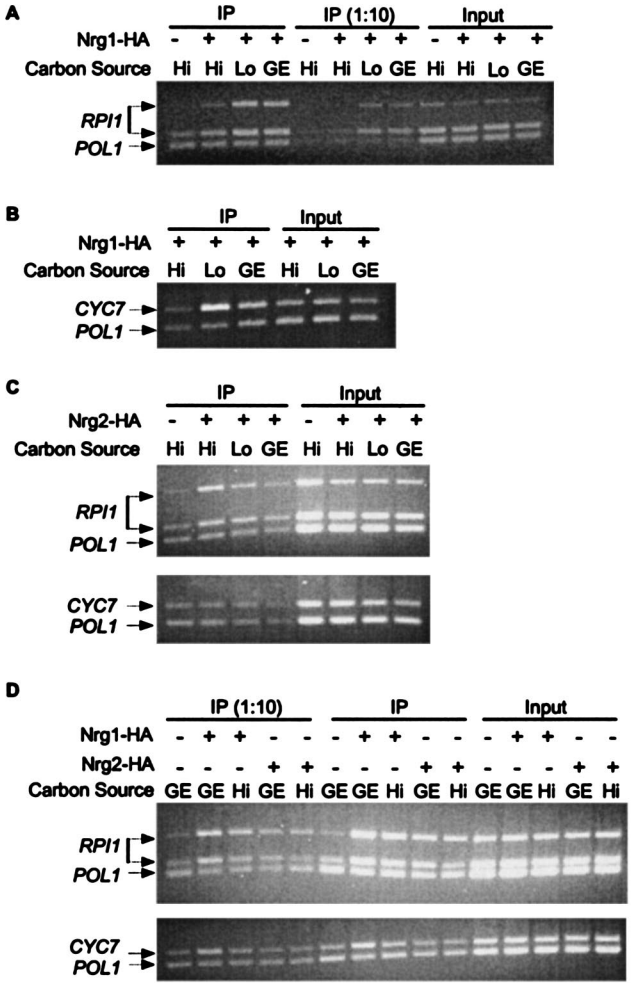

FIG. 3. Chromatin immunoprecipitation to detect Nrg1-HA and Nrg2-HA binding. Cells expressed Nrg1-HA or Nrg2-HA from the chromosomal locus (MCY4744 and MCY4751, respectively) or expressed native, untagged Nrg1 (MCY4702). (A to C) Cells were grown in YEP containing 2% glucose (Hi). Cells expressing HA-tagged Nrg proteins were also shifted to 0.05% glucose (Lo) for 3 h or were grown in 2% glycerol–2% ethanol (GE). (D) Cells were grown in YEP containing 2% glycerol–2%ethanol and were shifted to 2% glucose for 5 min. DNA was purified from total chromatin (input) and chromatin that was immunoprecipitated with anti-HA (IP). DNA from the input was diluted 1:1,000, and DNA from the immunoprecipitation was used undiluted or diluted 1:10, where indicated. DNA was also used at a 1:3 dilution to confirm linearity (data not shown). Five microliters was used as a template in PCRs with primers specific for the control *POL1* coding sequence and two regions of the *RPI1* promoter, spanning nucleotides −1610 to −1352 and −1137 to −697 (CCCCT sequences are at positions −1571, −1086, and −731) or the *CYC7* promoter (−480 to −188).

though we have not attempted to quantitate relative increases in protein levels and binding. The major point is that binding did not decrease.

We next examined binding of Nrg1-HA to the promoter of the *CYC7* gene. This gene is regulated by glucose repression (13) and showed increased expression in glucose-grown *nrg1*Δ *nrg2*Δ double mutant cells relative to the wild type in a microarray analysis (V. K. Vyas, unpublished data). DNA recovered by chromatin immunoprecipitation was amplified with primers spanning the region responsible for glucose repression (23), which includes three CCCCT sites. Binding of Nrg1 was higher in glucose-limited or glycerol-ethanol-grown cells than in glucose-grown cells (Fig. 3B). Thus, release of Nrg1-mediated glucose repression is not achieved by disrupting the binding of Nrg1 to DNA.

Finally, we assayed the binding of Nrg1-HA to the *FLO11* promoter, which is nearly identical to the *STA1* promoter (6) and includes a known Nrg1 binding site (22). Nrg1 and Nrg2 repress *FLO11* expression during growth in glucose, and *FLO11* RNA increases substantially following a shift to low glucose (10). Chromatin immunoprecipitation experiments were carried out using primers for the region −2200 to −1971, which encompasses the Nrg1 binding site. Analysis of the amplified DNA by gel electrophoresis indicated that binding to the *FLO11* promoter was relatively weak (data not shown), so we used quantitative PCR. As was the case for *RPI1* and *CYC7*, binding did not decrease but rather increased in the absence of glucose. The immunoprecipitation efficiencies, presented as the increase over the *POL1* coding sequence control, were 1.4-fold for glucose-grown cells, 3.0-fold following a 3-h shift to low glucose, and 2.9-fold for cells grown in glycerol-ethanol. Thus, release of Nrg1-mediated glucose repression of *FLO11* does not occur by release of Nrg1 from its binding site.

We further considered the possibility that Nrg1 binds to DNA to facilitate establishment of a repressive state but that its presence is not required for maintenance of this state, as has been suggested for the Mig1 repressor (5). To explore the possibility that Nrg1 binding transiently increases when cells are shifted from nonrepressing to glucose-repressing conditions, we grew cells in a nonfermentable carbon source, shifted them to 2% glucose for 5 min, and assessed binding by chromatin immunoprecipitation. However, binding to the *RPI1* and *CYC7* promoters did not increase (Fig. 3D).

Binding of Nrg2 to DNA. To assess the regulation of Nrg2 binding by glucose signals, we similarly assayed the binding of Nrg2-HA to the *RPI1* promoter by chromatin immunoprecipitation. In cells grown in high glucose, Nrg2-HA bound to both regions of the *RPI1* promoter and remained bound after a shift to conditions of glucose deprivation (Fig. 3C). Binding was also evident during growth in glycerol-ethanol and after a 5-min shift to high glucose (Fig. 3C and D). In contrast to Nrg1, Nrg2 did not exhibit increased binding in the absence of glucose, but rather, binding appeared to decrease. However, Nrg2 protein levels also decreased under these conditions (Fig. 2).

Using the same immunoprecipitated DNA, we were unable to detect binding of Nrg2-HA to the *CYC7* promoter, as judged by comparison with the untagged Nrg2 control, in two independent experiments (Fig. 3C and D). These findings may reflect a difference in the DNA-binding properties of Nrg1 and Nrg2.

Promoter-bound Nrg1, but not Nrg2, functions as an activator in mutant cells lacking the Ssn6-Tup1 corepressor. The Ssn6-Tup1 corepressor is required for repression by both Nrg1 (22) and Mig1, another repressor of glucose-regulated genes (27, 28). LexA-tagged Mig1 and Nrg proteins repress expression of a reporter with LexA binding sites in wild-type cells (22, 27, 28, 30); however, in *ssn6*Δ mutant cells, LexA-Mig1 activates transcription (27, 28). We therefore tested LexA-Nrg proteins for the ability to activate transcription in *ssn6*Δ mu-

TABLE 2. Transcriptional activation by LexA-Nrg1 in the *ssn6*Δ mutant[a]

Genotype[b]	Protein[b]	Glucose[c]	β-Galactosidase activity with no. of LexA sites[d]:	
			0	6
*ssn6*Δ	LexA-Nrg1	Hi	0.3	140
	LexA-Nrg1	Lo	0.1	160
	LexA-Nrg2	Hi	0.1	0.8
	LexA	Hi	0.2	10
Wild type	LexA-Nrg1	Hi	0.4	0.3
	LexA-Nrg1	Lo	0.1	0.1
	LexA-Nrg2	Hi	0.1	0.04
	LexA	Hi	0.2	3.6

[a] Reporter plasmids contained the *lacZ* gene under control of the *GAL1* promoter with UAS_G deleted (pLR1Δ1) (31) or replaced by 6 LexA binding sites (pSH18-18).

[b] Strains MCY1974 (*ssn6*Δ) and MCY829 (wild type) were transformed with reporters containing 0 or 6 LexA binding sites and plasmids expressing LexA-Nrg1, LexA-Nrg2, or LexA alone (pV43, pV44, or pEG202).

[c] Cultures were grown to mid-log phase in selective SC plus 2% glucose (Hi); glucose-grown cells were also shifted to 0.05% glucose (Lo) for 3 h, as indicated.

[d] β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller units. Values are averages for the results for 4 to 6 transformants. For values >1, standard errors were <15%. Values for LexA alone were comparable in high- and low-glucose conditions.

tant cells by using the reporter assay (Table 2). Both proteins functioned to confer glucose repression of *STA2-lacZ* in the *nrg1*Δ *nrg2*Δ mutant (data not shown). LexA-Nrg1 strongly activated the reporter in glucose-grown *ssn6*Δ cells (140 U of β-galactosidase activity), and no additional activation was observed after a shift to low (0.05%) glucose (Table 2). In contrast, LexA-Nrg2 did not display any activator function in the *ssn6*Δ mutant (<1 U); immunoblot analysis confirmed that the protein was stably expressed (data not shown). No activation was detected in wild-type cells, consistent with previous results (30). These findings provide further evidence that the two Nrg proteins have distinct properties.

DISCUSSION

The Nrg1 and Nrg2 repressors have very similar DNA-binding domains and exhibit a considerable degree of functional overlap with respect to their roles in the regulation of glucose-repressed genes. However, the two proteins differ in sequence outside their DNA-binding domains, with only 27% identity. We present evidence that these two repressors are indeed differentially regulated and functionally distinct.

First, we show that expression of the Nrg1 and Nrg2 repressors is differently regulated in response to carbon source. Differences are manifest at both the RNA and protein levels in the Σ1278b genetic background. We found that expression of *NRG1* RNA is glucose repressed, whereas that of *NRG2* is not; these results are in accord with levels observed during the diauxic shift (4) but differ from those of Park et al. (22). Autoregulation may be in part responsible for this transcriptional control, as Nrg1 binds to the *NRG1* promoter (14). Regulatory differences were also observed at the protein level. Nrg1 levels were elevated in response to glucose limitation or growth in nonfermentable carbon sources, in parallel with RNA levels. In contrast, levels of Nrg2 protein decreased in response to glucose limitation, despite nearly constant RNA levels. These differences in the regulation of expression of the two proteins in response to carbon source suggest that they have distinct physiological roles.

Other lines of evidence support the view that Nrg1 and Nrg2 are functionally distinct. First, LexA-tagged Nrg1 and Nrg2 behaved differently in a reporter assay, indicating that the two proteins have distinct properties; in mutant cells lacking the Ssn6-Tup1 corepressor, Nrg1 activated transcription of a reporter, whereas Nrg2 did not. Second, in two independent chromatin immunoprecipitation experiments, we easily detected binding of Nrg1 to the *CYC7* promoter but did not detect binding of Nrg2, suggesting that the two have different DNA-binding properties. Third, *nrg1*Δ and *nrg2*Δ mutations differentially affect adherence of cells to plastic, which is a *FLO11*-dependent process (C. D. Berkey, unpublished data). Finally, *nrg1*Δ and *nrg2*Δ mutations cause different phenotypes with respect to regulation of *STA2* expression (10, 29). The highly conserved promoter of the *STA* and *FLO11* genes is a bona fide target of Nrg1 and Nrg2 (10, 22, 29; this study) and exhibits differential regulation by Nrg1 and Nrg2 in response to glucose. The *nrg1*Δ mutation relieves the glucose repression of a *STA2-lacZ* promoter fusion more effectively than *nrg2*Δ and also enhances derepression to a greater extent (10, 29). Another pair of repressors with very similar zinc fingers, Mig1 and Mig2, also have largely redundant roles in glucose repression, but their relative affinities for various binding sites differ and their expression and function are regulated differently (16, 17).

One possible mechanism for regulating Nrg repressor function in response to glucose signals is through regulation of DNA binding. Chromatin immunoprecipitation studies indicated that glucose deprivation does not prevent the binding of Nrg1 to any of the three promoters tested, *RPI1*, *CYC7*, and *FLO11*. On the contrary, Nrg1 binding in cells shifted to limiting glucose or grown in nonfermentable carbon sources was elevated relative to that in glucose-grown cells, in accord with the higher Nrg1 protein levels. Similarly, binding of Nrg2 to the *RPI1* promoter was not abolished in the absence of glucose. We also detected no immediate increase in binding of either protein when cells grown in nonfermentable carbon sources were provided with glucose. These findings indicate that release of Nrg-mediated glucose repression is not achieved by disrupting the binding of the Nrg proteins to DNA, although it remains possible that at some promoters binding is regulated by the interactions of Nrg proteins with other glucose-responsive transcription factors. The simple interpretation of these findings is that glucose signals primarily regulate the repressor function of Nrg proteins by mechanisms other than their binding to DNA. Similarly, osmotic stress does not release Sko1-mediated repression by disrupting Sko1 binding (24).

The presence of the Nrg1 repressor at higher levels under nonrepressing conditions may facilitate rapid repression upon restoration of glucose. For example, if modification of the repressor is regulated by glucose signals, the presence of a large pool of unbound Nrg1 that is accessible to the modifying enzymes may facilitate the replacement of DNA-bound Nrg1 with Nrg1 exhibiting a modification state that reflects current

signals. Other repressors also are induced under nonrepressing conditions. Expression of a *MIG1-lacZ* promoter fusion is higher in the absence of glucose (16). The *CRT1* gene, which encodes a repressor that recruits Ssn6-Tup1 to DNA damage-inducible genes, is autoregulated and induced by DNA damage, and it was proposed that the increased levels of Crt1 facilitate a return of Crt1-regulated genes to the repressed state after the damage has been repaired (9).

The finding that LexA-Nrg1 activates transcription in *ssn6*Δ mutant cells raises the possibility that Nrg1 functions as an activator in wild-type cells in some contexts. Because the absence of glucose does not abolish DNA binding, release of glucose repression could entail both loss of repressor function and also involvement in activation. This is the case for the Sko1 repressor, which both represses and activates in conjunction with Ssn6-Tup1 (24), and Ssn6-Tup1 has been implicated in activation, as well as repression, of Mig1-repressed promoters (21). The presence of Nrg1 at promoters under nonrepressing conditions is compatible with a role in activation.

We have further explored the relationship between the Nrg proteins and Snf1 protein kinase. The increase in Nrg1 protein in response to glucose limitation required Snf1 kinase at the RNA level. Comparison of protein and RNA levels in *snf1*Δ mutant and wild-type cells suggested that Snf1 also positively regulates Nrg1 at the protein level; previous studies implicated Snf1 in stabilizing overexpressed Nrg proteins, and Snf1 protein, but not its catalytic activity, was required, consistent with the demonstrated physical interaction between Snf1 and Nrg proteins (30). The relationship between Snf1 kinase and the Nrg repressors is clearly complex because genetic evidence suggests that Snf1 negatively regulates Nrg function: Snf1 and the Nrg repressors act antagonistically with respect to *STA2* and *FLO11* expression, filamentous invasive growth, and initiation of biofilm formation (10, 29). Previous experiments yielded no evidence that Snf1 kinase directly phosphorylates Nrg1 (30), and we detected no phosphorylation of bacterially expressed Nrg1 or Nrg2 by Snf1 kinase purified from yeast (V. K. Vyas and G. Hovel-Miner, unpublished data).

The evidence presented here that Nrg1 and Nrg2 are distinct with respect to their regulation and function suggests that their regulatory roles in glucose repression are more complex than heretofore recognized. The differences in sequence outside their DNA-binding domains may allow them to interact differently with regulatory proteins or other transcription factors. The differences in expression and function of these two repressors, in combination with their highly similar DNA-binding domains, likely contribute to the complex and finely tuned regulation of the large array of glucose-repressed genes in the yeast genome.

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