Repressors Nrg1 and Nrg2 Regulate a Set of Stress-Responsive Genes in Saccharomyces cerevisiae

Valmik K. Vyas,1† Cristin D. Berkey,2 Takenori Miyao,2‡ and Marian Carlson1,2* Integrated Program in Cellular, Molecular and Biophysical Studies1 and Department of Genetics and Development,2 Columbia University, New York, New York

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The yeast Saccharomyces cerevisiae responds to environmental stress by rapidly altering the expression of large sets of genes. We report evidence that the transcriptional repressors Nrg1 and Nrg2 (Nrg1/Nrg2), which were previously implicated in glucose repression, regulate a set of stress-responsive genes. Genome-wide expression analysis identified 150 genes that were upregulated in nrg1Δ nrg2Δ double mutant cells, relative to wild-type cells, during growth in glucose. We found that many of these genes are regulated by glucose repression. Stress response elements (STREs) and STRE-like elements are overrepresented in the promoters of these genes, and a search of available expression data sets showed that many are regulated in response to a variety of environmental stress signals. In accord with these findings, mutation of NRG1 and NRG2 enhanced the resistance of cells to salt and oxidative stress and decreased tolerance to freezing. We present evidence that Nrg1/Nrg2 not only contribute to repression of target genes in the absence of stress but also limit induction in response to salt stress. We suggest that Nrg1/Nrg2 fine-tune the regulation of a set of stress-responsive genes.

To survive in natural environments, microorganisms must be able to adapt to changes in environmental conditions, including changes in nutrient availability, temperature, pH, osmolarity, and the presence of oxidative agents. One of the mechanisms by which microorganisms adapt is by changing their genomic expression programs (24, 40). The budding yeast Saccharomyces cerevisiae responds to environmental stress by rapidly altering the expression of sets of genes (5, 12). Each expression program is specific to the particular stress with respect to the genes affected, the magnitudes of the changes in RNA levels, and the temporal patterns of expression (12). However, a large set of genes responds similarly to many kinds of environmental change by what have been called the environmental stress response (ESR) (12) and the common environmental response (5), which involve a variety of environmental stress signals. In accord with these findings, mutation of NRG1 and NRG2 enhanced the resistance of cells to salt and oxidative stress and decreased tolerance to freezing. We present evidence that Nrg1/Nrg2 not only contribute to repression of target genes in the absence of stress but also limit induction in response to salt stress. We suggest that Nrg1/Nrg2 fine-tune the regulation of a set of stress-responsive genes.

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and MCY5326 by transformation. The strains (Open Biosystems, Huntsville, AL) were introduced into MCY5314 were made by transformation of strains containing Nrg1/Nrg2 limit the induction of Nrg-repressed genes in response to salt stress.

**Materials and Methods**

**Strains and genetic methods.** *S. cerevisiae* strains used in this study are listed in Table 1. The *gal83Δ* allele has been described previously (46). *msn2Δ::kanMX4* and *msn4Δ::kanMX4* alleles were recovered from genomic DNA of mutant strains (Open Biosystems, Huntsville, AL) and were introduced into MCYS314 and MCYS323 by transformation. The *natMX4* and *bphMX4* deletion alleles were made by transformation of strains containing *kanMX4* or *kanMX6* alleles with DNA containing the *natMX4* or *bphMX4* cassettes, respectively (14). Rich medium was yeast extract-peatone-potassium (YPD) containing 2% glucose (YPD) or the indicated carbon source (38). Cells were grown at 30°C unless otherwise specified.

**Preparation of RNA.** Cells (50 ml) were grown to an optical density at 600 nm of 1 in YPD and collected by filtration, except that for shifts to low glucose, cells were collected by centrifugation, washed, resuspended in YEP plus 0.05% glucose for the indicated time, and collected by centrifugation. Cells were resuspended in 0.7 ml of TES buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 0.5% sodium dodecyl sulfate) and frozen in liquid nitrogen. An equal volume of acid phenol was added to each sample. Samples 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 equal volumes of phenol and then extracted twice with chloroform. RNA was precipitated with ethanol and resuspended in water.

**Gene expression analysis.** Total RNA (30 μg) was used as a template for cDNA synthesis with the T7-(dT)24 oligonucleotide, as described by Affymetrix (Santa Clara, CA). Biotin-labeled cRNA was transcribed using an Enzo BioArray high-yield transcript-labeling kit (Affymetrix), fragmented, and hybridized to Affymetrix yeast genome S98 arrays using the manufacturer’s protocols. Absolute and comparative analyses of expression data were performed with Affymetrix Microarray Suite 5.0, following the manufacturer’s guidelines and using the default settings. For each strain and condition, samples were prepared in duplicate. All pairwise comparisons between mutant and wild-type (WT) samples were analyzed, yielding four signal log ratios (SLRs; base 2) for each mutant-to-WT comparison, which were exported into Microsoft Excel and then averaged. All comparisons were between strains with matched auxotrophs, except MCY4710 (*nrg1Δ*), which is His+. Genes that showed an average SLR of 1 (twofold difference) and that also displayed significant changes in at least two of these comparisons (as measured by the SLR P value determined by Affymetric Microarray Suite 5.0) were kept for further analysis. Data from probe sets not conforming to standard open reading frame nomenclature guidelines (6) were not included in subsequent analysis. Filtered datasets were exported into Cluster and Treeview (10), where they were K-means clustered and visualized. Original data are available in Tables S1, S2, and S3 in the supplemental material.

**Northern blot analysis.** RNAs (40 μg) were separated by electrophoresis on a 1.2% agarose-MOPS (morpholinepropanesulfonic acid) gel containing formaldehyde. RNAs were transferred to Hybond N+ membrane (Amersham Biosciences). Probes contained ~250 to 1,500 bp of coding sequence and were 32P labeled using Ready-To-Go DNA-labeling beads (Amersham Biosciences). Levels of RNA relative to those of loading controls were quantified with a Molecular Dynamics PhosphorImager (Amersham Biosciences) and ImageQuant software (1). The GSY1 probe did not cross-hybridize with GSY2.

**Results**

**Whole-genome expression analysis of *nrg1Δ* and *nrg2Δ* single and double mutants.** To assess the roles of *Nrg1/Nrg2* in genomic expression, we prepared RNAs from wild-type, *nrg1Δ*, *nrg2Δ*, and *nrg1Δ nrg2Δ* cells (Σ207b8 genetic background) grown to mid-log phase in rich medium containing 2% glucose. The growth rates of the wild-type and double mutant strains were identical in three independent experiments (data not shown). RNAs were analyzed by hybridization to a whole-genome DNA oligonucleotide microarray, and measurements of RNA levels were organized by clustering (10) to group genes according to their similarity in terms of expression pattern. Expression of 150 genes was increased twofold or more in the *nrg1Δ nrg2Δ* double mutant relative to that in the wild type, and RNA levels of 41 genes were increased at least threefold (Fig. 1; see Table S1 in the supplemental material). Surprisingly, most of these genes (119) also showed increased expression (>1.5-fold) in each single mutant, indicating that both *Nrg1* and *Nrg2* are required for full repression; only 11 genes showed <1.5-fold-increased expression in both single mutants, indicating that either *Nrg1* or *Nrg2* is sufficient for repression. Thus, *Nrg1* and *Nrg2* repress highly overlapping sets of genes, consistent with the strong similarity of their DNA-binding domains (84% identity). It is possible that *Nrg1/Nrg2* regulate some of these genes by indirect mechanisms.

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| MCY4521 | MATa nrg1Δ::kanMX6 ade2-101 his3Δ200 leu2-1,122 lys2-801 trp1Δ1 ura3-52 | 47 |
| MCY4523 | MATa nrg2Δ::kanMX6 ade2-101 his3Δ200 leu2-1,122 lys2-801 trp1Δ1 ura3-52 | 47 |
| MCY4525 | MATa ade2-101 his3Δ200 leu2-1,122 lys2-801 trp1Δ1 ura3-52 | 47 |
| MCY4549 | MATa nrg1Δ::kanMX6 nrg2Δ::HistMX6 ade2-101 his3Δ200 leu2-1,122 lys2-801 trp1Δ1 ura3-52 | 47 |
| MCY4555 | MATa his3Δ leu2Δ | 46 |
| MCY4569 | MATa his3Δ leu2Δ ura3Δ reg1Δ::URA3 | 46 |
| MCY4571 | MATa his3Δ leu2Δ ura3Δ reg1Δ::URA3 gal83Δ | 46 |
| MCY4577 | MATa leu2Δ ura3Δ | This study |
| MCY4706 | MATa nrg2Δ::His3MX6 his3Δ leu2Δ ura3Δ | 46 |
| MCY4710 | MATa nrg1Δ::kanMX6 his3Δ leu2Δ ura3Δ | 46 |
| MCY4714 | MATa nrg1Δ::kanMX6 nrg2Δ::HistMX6 his3Δ leu2Δ ura3Δ | 46 |
| MCYS314 | MATa ade2-101 his3Δ200 leu2-1,122 lys2-801 trp1Δ1 ura3-52 | This study |
| MCYS323 | MATa msn2Δ::natMX4 msn4Δ::hphMX4 ade2-101 his3Δ200 leu2-1,122 lys2-801 trp1Δ1 ura3-52 | This study |
| MCYS326 | MATa his3Δ leu2Δ ura3Δ | This study |
| MCYS338 | MATa msn2Δ::kanMX6 msn4Δ::natMX4 his3Δ leu2Δ ura3Δ | This study |
| MCYS378 | MATa nrg1Δ::bphMX4 nrg2Δ::His3MX6 his3Δ leu2Δ ura3Δ | This study |
| MCYS385 | MATa msn2Δ::kanMX4 msn4Δ::natMX4 nrg1Δ::bphMX4 nrg2Δ::His3MX6 his3Δ leu2Δ ura3Δ | This study |
| MCYS414 | MATa nrg1Δ::kanMX6 nrg2Δ::HistMX6 ade2-101 his3Δ200 leu2-1,122 lys2-801 trp1Δ1 ura3-52 | This study |
In addition, expression of 265 genes was decreased at least twofold in the \textit{nrg1}/\textit{nrg2} mutant, and 187 genes showed decreased expression in each single mutant (see Table S2 in the supplemental material). These findings suggest that Nrg1/Nrg2 play positive regulatory roles either indirectly, by Nrg1/Nrg2-mediated repression of genes encoding repressors, or directly. The possibility that Nrg1 directly activates some promoters, as do other repressors (32, 34), was raised by evidence that, in mutant cells lacking the Ssn6-Tup1 corepressor, DNA-bound LexA-Nrg1 activated transcription of a reporter (2). However, previous studies have characterized Nrg1/Nrg2 as repressors (21, 22, 33, 39, 47, 49), and we have therefore focused on the 150 genes that were upregulated in the mutant.

Northern blot analysis of representative genes yielded results parallel to those of the microarray analysis (Fig. 2 and data not shown). For \textit{GSY1}, \textit{PTR2}, \textit{PGU1}, \textit{PHO84}, \textit{RPI1}, and \textit{INH1}, RNA levels were similarly increased in the single and double mutants relative to the loading control level, confirming that both Nrg1 and Nrg2 are necessary for full repression. For \textit{SGA1}, \textit{ENA}, and \textit{RSB1}, RNA levels in the double mutant were substantially higher than that in either single mutant, confirming that either Nrg1 or Nrg2 alone confers repression. Finally, RNA levels of \textit{CTS1}, a representative downregulated gene, were decreased in the mutants.

\textbf{Functions of Nrg-repressed genes.} Previous studies implicated Nrg1 and/or Nrg2 in glucose repression, haploid invasive and diploid pseudohyphal growth, adaptation to alkaline pH, and ion tolerance (21, 22, 33, 47, 49). In accord with these findings, the Nrg1/Nrg2-repressed (Nrg-repressed) genes identified in this study are involved in mitochondrial function, carbon utilization and signaling, nitrogen utilization, cell wall and cell surface function, transcriptional control, mating, transport of nutrients and ions, and other cellular processes (Table 2). The largest category comprises genes involved in mitochondrial function, many of which are regulated in response to the carbon source. \textit{IMP2}, \textit{NCE102}, \textit{PUT4}, \textit{XBP1}, and \textit{YOR161C} are important for invasive or pseudohyphal growth (28, 43). Mutation of the \textit{ENA} locus, \textit{PHO84}, or \textit{SHC1} impairs growth at alkaline pHs (13, 16, 17). Many of the Nrg-repressed genes are important for growth on media containing NaCl or sorbitol, including not only the \textit{ENA} genes but also \textit{AGA2}, \textit{ADK2}, \textit{IMP2}, \textit{NCE102}, \textit{PUT4}, \textit{XBP1}, and \textit{YOR161C}.
TABLE 2. Functional categorization of Nrg-repressed genes

| Category                  | Gene(s)*                                                                 |
|---------------------------|--------------------------------------------------------------------------|
| Mitochondria              | ADK2, ALD4, ATP15, ATP16, ATP17, ATP18, ATP2, ATP20, ATP4, ATP5, COX16, COX4, COX5A, COX5B, COX6, COX7, CRC1, CYB2, CYC1, CYC2, EM5, FMC1, FMP16, IDH1, IMP2, INH1, LSP1, MCR1, MRPL33, MRPL7, MRPS17, MRPS18, NDE1, NDL1, NGI1, ODC1, PET177, QCR2, RIP1, RPM2, RMS10, SDP1, TUF1, YDR031W, YFR011C, YGL226W, YHR080C, YIL087C, YMR030W, YMS1, YMR157C, YMR252C, YNL200C, YNL274C, YOR161C, YPL183W-A |
| Carbon utilization/signaling | GAL7, GLC2, GST1, HXK1, HXK2, MTH1, PEX2, PGM2, PGU1, PYK2, SGA1          |
| Nitrogen utilization/signaling | AGP2, DAL5, PRT2, PUT4, TAT1, YBR147W, YGR053C, YHR033W, YPL113C          |
| Cell wall/surface          | LRG1, PDR11, PRY1, PRY3, RSB1, SHC1, TIP1, WSC4, YLR042C, ZPS1            |
| Cell cycle                 | AGA2, ASG7, FUS1, MGD1, MFA1, PRM1                                         |
| Matting                    | CTR3, CTL1, ENA, GAD                                                        |
| Transcription factors      | CUP9, FHT1, XBP1, YPL500W, ZMS1                                           |
| Ribosome                   | RPL22B, RPL8A, RRM3                                                        |
| Cell cycle                 | CLB1                                                                      |
| Other                      | ELO1, FRE4, GSP2, NCE102, PH084, YMR194C-A                                 |

* Functional information was available for 112 of the 150 genes. Asterisks indicate that the expression of the gene was confirmed to be upregulated in the nrg1Δ nrg2Δ mutant by Northern blot analysis. Underlined genes were classified as part of the ESR (12). Expression of ALD4 was also increased but not sufficiently to meet the criteria for inclusion on this list. Σ1278b contains a single ENS gene (48).

Nrg1/Nrg2 regulate carbon source-responsive genes. Previous work showed that Nrg1/Nrg2 contribute to repression of various glucose-repressed genes (21, 33, 47, 49). To determine whether the Nrg-repressed genes are in general regulated by glucose repression, we carried out genome expression analyses of wild-type cells that were grown in high (2%) glucose and low glucose. Most of the Nrg-repressed genes that were upregulated in the wild type were similarly upregulated in gal83Δ cells (data not shown); however, previous work has shown that upregulation is usually complex, involving both relief of repression and activation. We then asked whether the increased gene expression in glucose-grown regΔ cells relative to that in wild-type cells depends on Snf1-Gal83. Of the 90 genes that were upregulated in the regΔ mutant, expression of 89 was less elevated in the reg1Δ gal83Δ mutant (Fig. 1), suggesting that Snf1-Gal83 and Nrg1/Nrg2 regulate overlapping sets of genes.

Many Nrg1/Nrg2-repressed genes contain STREs and STRE-like sequences. To analyze sequence elements in the promoters of the 150 Nrg-repressed genes, we used regulatory sequence analysis tools to retrieve sequences 600 bp upstream of the ATG. Motifs were found using the oligo-analysis program with the default settings (45). The STRE sequence, C_aT, was identified most frequently and is present 131 times in 73 genes, and C_aT was also frequent (Fig. 3; see Table S4 in the supplemental material). CaNrg1 binds C_aT in vitro, and many C. albicans genes that are regulated by CaNrg1 carry a related sequence with the consensus (A/C) (A/G) C_aT (30). This consensus sequence is present 156 times in the promoters of 93 Nrg-repressed genes (Fig. 3), and a recent study showed that Nrg1 binds in vitro to an oligonucleotide containing AAC_aT (39). We note that the Nrg1 footprint at the STA1 promoter (33) includes both C_aT and ATC_aT. The closely related sequence GGaC_aT was identified as a consensus binding site for Nrg1 by genome-wide location analysis (15) and is present 25 times in 23 of the 150 Nrg-repressed genes. Together, these data suggest that Nrg1/Nrg2 bind STRE-like sequences.

The presence of STRE sites raised the possibility that the upregulation of these genes reflects a stress response triggered by reduced fitness of the nrg1Δ nrg2Δ mutant cells. Three lines of evidence suggest that this is not the case. First, the mutant cells exhibited a wild-type growth rate under the conditions employed. Second, only 25 of the 150 upregulated genes and 28 of the 265 downregulated genes were classified as part of the ESR (12). Third, some of the genes that were upregulated in...
the mutant are in fact downregulated in wild-type cells in response to stress (Fig. 3).

**Regulation of Nrg1/Nrg2-repressed genes in response to stress.** The presence of STRE sites and the regulation of many Nrg1/Nrg2 target genes in response to glucose limitation, which can be viewed as carbon stress, suggested that these genes may be broadly regulated in response to stress signals. To address this possibility, we combined our data with data sets derived from microarray analyses of gene expression under different environmental conditions (5, 9, 12) and clustered the Nrg-repressed genes with respect to their expression patterns (Fig. 3; also see Table S3 in the supplemental material).

Figure 3 further supports the view that the Nrg-repressed genes are regulated by carbon availability. Many of these genes were induced during the diauxic shift, when cells shift from fermentative to respiratory growth in response to the depletion of glucose, and during entry into stationary phase (clusters I and II). These genes were also more highly expressed during growth in less-preferred carbon sources (ethanol, galactose, and raffinose) than during growth in more-preferred carbon sources (glucose, mannose, and sucrose). In contrast, genes that were downregulated in response to glucose limitation (cluster III) were also correspondingly repressed under other conditions of poor carbon availability.

Many of the 150 Nrg-repressed genes were also regulated in response to multiple stresses, including nitrogen depletion, oxidative stress, various transitions to heat, cold shock, salt and osmotic stress, and both alkaline and acidic pHs. The genes in cluster I, which were strongly upregulated in response to glucose limitation, were also induced in response to all of these
different stress conditions, except cold shock. The genes in cluster II, which were also upregulated in response to glucose limitation, were less strongly affected by other stresses, except for an initial marked downregulation in response to salt and osmotic stress. The genes in cluster III were generally downregulated in response to glucose limitation and other environmental changes. Thus, the Nrg-repressed genes are regulated by stresses unrelated to carbon availability and exhibit three broadly different patterns of response to environmental change.

Evidence indicates that many of the 150 Nrg-repressed genes are also targets of Msn2/Msn4. The induction of 78 of these genes by acidic pH depends on Msn2/Msn4 (Fig. 3; columns at right); of the 91 genes that were upregulated at both the 10-min and 20-min time points in wild-type cells, 29 were less upregulated in the msn2Δ msn4Δ mutant, and 49 were downregulated, suggesting that Msn2/Msn4 function to overcome transcriptional repression (5). In addition, 106 of the Nrg-repressed genes were induced to some extent by overexpression of Msn2 and/or Msn4 (12).

**Regulation of NRG1/NRG2 expression by stress.** Our genomic expression analysis showed a 4.3-fold increase in *NRG1* RNA levels and a slight elevation in *NRG2* genes during a 1-h shift from high to low glucose (Fig. 3, top rows). These results are in accord with previous evidence that *NRG1* RNA levels, and Nrg1 protein levels, are elevated in response to glucose limitation or growth in nonpreferred carbon sources, while *NRG2* RNA levels remain nearly constant (2, 9, 12).

Expression of *NRG1* is also regulated in response to other stresses, and *NRG1* RNA levels showed a pattern broadly similar to those of the genes in cluster I, with particularly strongly increased expression upon the addition of salt or sorbitol (5, 12) (Fig. 3, top row). The downregulation of *NRG1* in response to alkaline pH represents a difference from the pattern in cluster I but is in accord with another report (22). We confirmed the regulation of *NRG1* RNA in response to salt stress by Northern blot analysis; *NRG1* RNA levels were elevated relative to that of the loading control, with similar results for the *S. cerevisiae* strain 1278b, W303, and S288C genetic backgrounds (Fig. 4 and data not shown). In addition, *NRG1* RNA was induced by salt stress and glucose limitation in the *msn2Δ msn4Δ* mutant (Fig. 4 and data not shown).

*NRG2* RNA levels showed only modest changes during exposure to stress conditions (5, 12) (Fig. 3). Northern blot analysis confirmed a very modest response to salt stress (data not shown).

**Nrg1/Nrg2 limit induction of target genes in response to salt stress.** To assess the effects of Nrg1/Nrg2 on gene expression during salt stress, we first examined the regulation of three Nrg-repressed, stress-induced genes, *CTT1*, *CYC7*, and *RSB1*. Nrg1 binds directly to the *CYC7* and *RSB1* promoters (2, 15), and osmotic induction of *CTT1* and *CYC7* depends on Msn2/Msn4 (35). We carried out Northern blot analysis of RNAs prepared from cells (1278b background) exposed to 1 M NaCl (Fig. 4A). Basal expression and induction of all three RNAs were increased in the *nrg1Δ nrg2Δ* mutant relative to that in the wild type, and upregulation of *NRG1* showed a similar temporal pattern. Induction was reduced in the *msn2Δ msn4Δ* mutant and was partially restored in the *msn2Δ msn4Δ nrg1Δ nrg2Δ* quadruple mutant.

In 1278b strains, the response to 1 M NaCl occurred much more slowly than in other characterized strains. Exposure of 1278b strains to 75 mM NaCl elicited a more rapid response (Fig. 4B [note that time points are different from those in Fig. 4A]). In addition to *CYC7*, we examined *SGA1* and *ENA* (1278b has only one *ENA* gene [48]), which were independently identified as Nrg1 targets by genome-wide location analysis (15). All three showed not only increased basal expression but also increased upregulation in the *nrg1Δ nrg2Δ* mutant relative to that in the wild type.

We next examined the regulation of several Nrg-repressed, stress-induced genes in W303 cells, which respond rapidly to salt stress. Induction of *XBP1*, *SGA1*, and *CTT1* in response to 0.5 M NaCl was increased in the *nrg1Δ nrg2Δ* mutant relative to that in the wild type (Fig. 4C). In this experiment, the temporal pattern of transient induction was evident and was unperturbed in the mutant. To further assess the role of Nrg1/Nrg2 in tempering the amplitude of induction, we exposed W303 cells to a mild stress, 0.3 M NaCl. The induction response was very brief, and the presence of Nrg1/Nrg2 reduced induction, particularly in the case of *SGA1* (Fig. 4D). Thus, these studies support a role for Nrg1/Nrg2 in limiting the amplitude of induction during the salt stress response.

With respect to the Nrg1/Nrg2 target genes that are repressed during exposure to stress (Fig. 3, cluster III), the simple model is that Nrg1/Nrg2 contribute to the repression response. We examined the regulation of one of these genes, *TIP1*, in the *nrg1Δ nrg2Δ* mutant during exposure to salt. Although the overall level of expression was higher in the mutant than in the wild type, the absence of Nrg1/Nrg2 did not prevent the transient repression response (Fig. 4C) (confirmed by quantitative analysis of the phosphorimaging data).

**Altered resistance of nrg1Δ nrg2Δ mutant cells to salt and to oxidative and cold stresses.** To assess the physiological importance of Nrg1/Nrg2 in stress responses, we tested *nrg1Δ nrg2Δ* mutant cells for the ability to tolerate several different stress conditions. Previously, the *nrg1Δ* mutant was reported to be salt tolerant (22). Both single and double mutants of the S288C genetic background grew better than the wild type on medium containing 1 M NaCl (Fig. 5A). Similarly, the *nrg1Δ nrg2Δ* mutant of the 1278b background showed increased salt tolerance relative to the wild type (Fig. 5B), although the latter cells were sensitive to 150 mM NaCl (1278b cells have defects at the *ENA* locus [48]). These findings are consistent with evidence that Nrg1/Nrg2 repress a number of genes, including *ENA* genes, that are induced by salt stress and contribute to salt resistance.

Many Nrg-repressed genes are induced by oxidative stress, and we therefore tested *nrg1Δ nrg2Δ* cells for altered resistance to oxidative stress. Cells were spread on a plate and exposed to a disk containing NaAsO$_2$ or H$_2$O$_2$. Both oxidative agents caused a larger zone of growth inhibition for wild-type cells than for *nrg1Δ nrg2Δ* cells (1278b background) (Fig. 5C).

The ability of yeast cells to tolerate low or freezing temperatures depends on the induction of trehalose synthesis (20). Although we did not identify genes for trehalose synthesis in this study, three of the Nrg-repressed genes, *PGM1*, *GSY1*, and *GLC3*, are important for the synthesis and metabolism of glycogen, another reserve carbohydrate. These three genes are among the many Nrg-repressed genes that are repressed in
response to cold shock (12). We therefore tested \textit{nrg1} \textit{nrg2} cells for survival at freezing temperatures by use of the assay of Kandror et al. (20). The mutant cells showed reduced ability to survive exposure to \(-20^\circ\text{C}\). After 10 days, 26\% of wild-type cells (\textit{S288C} background) remained viable, compared to 2.1\% of the mutant cells (Fig. 5D, top panel); similar results were obtained for cells of the \textit{\Sigma1278b} background, with 8.0\% viability for the wild type (MCY5326) and 1.6\% for the \textit{nrg1} \textit{nrg2} mutant (MCY5378). Survival seems to be dependent on the particular conditions for freezing; cells of the \textit{S288C} background showed much-reduced viability in an independent experiment using different tubes and a different freezer, although again the \textit{nrg1} \textit{nrg2} cells showed viability that was much reduced relative to that of wild-type cells (Fig. 5D, bottom panel). This increased susceptibility to freezing temperatures is consistent with evidence that, prior to freezing, the \textit{nrg1} \textit{nrg2} mutant exhibits elevated expression of genes that are normally repressed in response to cold shock.

**DISCUSSION**

By analysis of whole-genome expression, we have identified a set of 150 genes that were upregulated in the \textit{nrg1} \textit{nrg2} double mutant, relative to wild type, when cells were grown in rich medium containing glucose. These genes are involved in mitochondrial function, carbon utilization and signaling, nitrogen utilization, transport of nutrients and ions, cell wall and cell surface function, transcriptional control, mating, and other cellular processes. Of the 150 genes, 94 are glucose repressed and show increased expression when cells are limited for glucose, in accord with studies implicating Nrg1/Nrg2 in glucose repression of several specific genes (21, 33, 47, 49). Analysis of
the upstream regions of the Nrg1/Nrg2 targets revealed that STRE and STRE-like elements are overrepresented, and a search of available expression data sets showed that many of these genes are regulated by a variety of stresses and by the stress-responsive transcriptional activators Msn2/Msn4. Thus, these studies implicate Nrg1/Nrg2 in regulation of a set of stress-responsive genes.

We also note that Nrg1/Nrg2 may regulate a set of stress-responsive genes larger than that identified here. Our genomic expression analysis identified genes that, in the absence of Nrg1/Nrg2, exhibit increased expression during exponential growth in rich medium containing glucose (unstressed conditions). It seems likely that some, and perhaps many, Nrg1/Nrg2 target genes are activated primarily by stress-responsive activators and are simply not activated under such optimal growth conditions, despite release of Nrg-mediated repression. Thus, it is possible that Nrg1/Nrg2 repress additional stress-responsive genes. It is also possible that the effects of Nrg1/Nrg2 on some of the 150 genes identified in this study are indirect. Only 14 of the 150 genes were among the 128 targets of Nrg1 identified by genome-wide location analysis (P value < 0.001) carried out in cells exposed to hydrogen peroxide (SGA1, GSY1, RPI1, SMC1, PRY3, NDH1, FRE4, GAL7, IDH2, ELO1, ICY1, RSB1, YHR033W, and YGR050C) (15). However, there are at least two possible explanations for this minimal overlap: our study was carried out in unstressed cells, and the binding of Nrg1 to some promoters may not be easily detected by genome-wide location analysis. In accord with this view, the 128 identified targets did not include three genes shown to bind Nrg1 (CYC7, DIT1, and FLO11, which has an upstream region nearly identical to that of STA1; see references 2, 3, and 39).

The relationship of Nrg1/Nrg2 to Msn2/Msn4 is not clear. Many Nrg-repressed genes contain STRE and STRE-like elements. The footprint of Nrg1 in the STA promoter (33) and the binding of CaNrg1 to the STRE (30) suggest that the binding specificity of Nrg1/Nrg2 overlaps that of Msn2/Msn4, and it is possible that Nrg1/Nrg2 compete directly with Msn2/Msn4 for binding to some promoters. The individual roles of Nrg1 and Nrg2 are also unclear. For most of the 150 Nrg-repressed genes, RNA levels in glucose-grown nrg1Δ and nrg2Δ single mutants were similar to those of the double mutant, indicating that both Nrg1 and Nrg2 are required for full repression under unstressed conditions. Levels of the two proteins are similar in unstressed cells (in both wild types and single mutants; C. D. Berkey, unpublished data), and twofold-reduced levels of total Nrg1/Nrg2 protein may be insufficient for repression at most promoters. In response to stress, the level of NRG1 RNA increases more than that of NRG2 (2, 5, 12); this differential regulation suggests that Nrg1 is particularly important for adaptation to stress.

The phenotypes of mutants lacking Nrg1 and/or Nrg2 support the physiological importance of these repressors in the regulation of stress responses. Previous studies showed that the nrg1Δ mutation increases ion tolerance and suppresses the rim101Δ defect in alkaline pH tolerance (22) and that the nrg1Δ nrg2Δ mutant exhibits increased invasive and pseudohyphal growth in response to carbon and nitrogen limitation, respectively (21). Here we have documented increased resistance to salt and oxidative stress, consistent with the increased expression of stress-induced genes in the mutants. Conversely, we showed that the nrg1Δ nrg2Δ mutant is more sensitive to freezing temperatures, in accord with evidence that the mutant exhibits increased expression of genes that are repressed in response to cold shock.
We have examined the expression of selected Nrg-repressed, stress-induced genes in the nrg1Δ nrg2Δ mutant during salt stress. In the cases of CTT1, CYC7, RSB1, ENA, XBP1, and SGA1, the RNA was upregulated more strongly in the mutant than in the wild type, indicating that Nrg1/Nrg2 limit the amplitude of induction, presumably by counteracting the function of stress-responsive activators. Based on our initial identification of Nrg-repressed genes by virtue of their increased expression in glucose-grown nrg1Δ nrg2Δ cells, Nrg1/Nrg2 clearly function to keep some stress-induced genes securely off in the absence of stress. It is also possible that the repressive function of Nrg1/Nrg2 effectively raises the threshold for induction of the stress response.

Nrg1/Nrg2 alter the resistance of cells to various stresses in growth assays, and yet these repressors make only modest contributions to the regulation of the genes we have examined here. Although Nrg1/Nrg2 may play more substantial roles in controlling expression of other genes, or in responses to other stresses, another possibility is that subtle regulatory effects matter to the cell. Stress responses typically involve induction of large numbers of genes, and large energetic costs are associated with such a gene expression program. The contribution of Nrg1/Nrg2 to fine-tuning the regulation of stress-responsive genes may serve to minimize unnecessary energy expenditures and enhance competitiveness in natural environments.

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REFERENCES

1. Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image processing with Image. J. Biophoton. Int. 1136–42.
2. Berkley, C. D., V. K. Vyas, and M. Carlson. 2004. Nrg1 and Nrg2 transcriptional repressors are differentially regulated in response to carbon source. Eukaryot. Cell 3:311–317.
3. Boy-Marcotte, E., M. Perrot, F. Bussereau, H. Boucherie, and M. Jacquet. 1998. Msn2p and Msn4p control a large number of genes induced at the transcriptional level which are repressed by cyclic AMP in Saccharomyces cerevisiae. J. Bacteriol. 180:1044–1052.
4. Braun, B. R., D. Kadosh, and A. D. Johnson. 2001. NRG1, a repressor of filamentous growth in C. albicans, is down-regulated during filament induction. EMBIO J. 17:473–486.
5. Causton, H. C., B. Ren, S. S. Koh, C. T. Harbison, E. Kanin, E. G. Jennings, T. I. Lee, H. L. True, E. S. Lander, and R. A. Young. 2004. Transcriptional regulatory code of a eukaryotic genome. Nature 431:99–104.
6. Haro, R., B. Garcia-de-la-Peña, and J. M. Rodriguez-Navarro. 1991. A novel pTye ATPase from yeast involved in sodium transport. FEBS Lett. 291:189–191.
7. Hong, S. K., S. B. Hwa, M. Snyder, and E. Y. Choi. 1997. SHC1, a high pH inducible gene required for growth at alkaline pH in Saccharomyces cerevisiae. Biochem. Biophys. Res. Commun. 255:116–122.
8. Jamieson, D. J. 1998. Oxidative stress responses of the yeast Saccharomyces cerevisiae. Yeast 14:1511–1543.
9. Kadosh, D., and A. D. Johnson. 2005. Induction of the Candida albicans filamentous growth program by relief of transcriptional repression: a genome-wide analysis. Mol. Biol. Cell 16:2903–2912.
10. Kadow, R., O. N. Bresch, F. Kreysdlin, D. Cavaleri, and A. L. Goldberg. 2004. The yeast HOG MAPK pathway for protein export in Saccharomyces cerevisiae. J. Biol. Chem. 279:22273–22277.
11. Kaplan, D., R. S. Belkin, R. Benito, S. Brachat, S. Campanaro, M. Curini, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Goldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, S. L. Kelly, A. P. Arnkin, A. Aström, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curini, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J.

12. Garfinkel, M. Gerstein, D. Gotte, U. Goldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, S. L. Kelly, A. P. Arnkin, A. Aström, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curini, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J.
Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J. Biol. Chem. 275:8290–8300.

36. Rep, M., V. Reiser, U. Gartner, J. M. Thevelein, S. Hohmann, G. Ammerer, and H. Ruis. 1999. Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msn1p and the novel nuclear factor Hot1p. Mol. Cell. Biol. 19:5474–5485.

37. Reynolds, T. B., and G. R. Fink. 2001. Bakers' yeast, a model for fungal biofilm formation. Science 291:878–881.

38. Rose, M. D., F. Winston, and P. Hieter. 1990. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

39. Rothfels, K., J. C. Tanny, E. Molnar, H. Friesen, C. Commissio, and J. Segall. 2005. Components of the ESCRT pathway, DFG16, and YGR122w are required for Rim101 to act as a corepressor with Nrg1 at the negative regulatory element of the DIT1 gene of Saccharomyces cerevisiae. Mol. Cell. Biol. 25:6772–6788.

40. Ruis, H., and C. Schuller. 1995. Stress signaling in yeast. BioEssays 17:959–965.

41. Schmitt, A. P., and K. McEntee. 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 93:5777–5782.

42. Sickmann, A., J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H. E. Meyer, B. Schonfisch, I. Perschil, A. Chacinska, B. Guiard, P. Rehling, N. Pfanner, and C. Meisinger. 2003. The proteome of Saccharomyces cerevisiae mitochondria. Proc. Natl. Acad. Sci. USA 100:13207–13212.

43. Suzuki, C., Y. Hori, and Y. Kashiwagi. 2003. Screening and characterization of transposon-insertion mutants in a pseudohyphal strain of Saccharomyces cerevisiae. Yeast 20:407–415.

44. Tu, J., and M. Carlson. 1995. REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae. EMBO J. 14:5939–5946.

45. van Helden, J. 2003. Regulatory sequences analysis tools. Nucleic Acids Res. 31:3593–3596.

46. Vyas, V. K., S. Kuchin, C. D. Berkey, and M. Carlson. 2003. Snf1 kinases with different β-subunit isoforms play distinct roles in regulating haploid invasive growth. Mol. Cell. Biol. 23:1341–1348.

47. Vyas, V. K., S. Kuchin, and M. Carlson. 2001. Interaction of the repressors Nrg1 and Nrg2 with the Snf1 protein kinase in Saccharomyces cerevisiae. Genetics 158:563–572.

48. Wieland, J., A. M. Nitsche, J. Strayle, H. Steiner, and H. K. Rudolph. 1995. The PMR2 gene cluster encodes functionally distinct isoforms of a putative Na+/H+ pump in the yeast plasma membrane. EMBO J. 14:3870–3882.

49. Zhou, H., and F. Winston. 2001. NRG1 is required for glucose repression of the SUC2 and GAL genes of Saccharomyces cerevisiae. BMC Genet. 2:5.