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Nrg1 Is a Transcriptional Repressor for Glucose Repression of STA1 Gene Expression in Saccharomyces cerevisiae

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Expression of genes encoding starch-degrading enzymes is regulated by glucose repression in the yeast *Saccharomyces cerevisiae*. We have identified a transcriptional repressor, Nrg1, in a genetic screen designed to reveal negative factors involved in the expression of *STA1*, which encodes a glucoamylase. The *NRG1* gene encodes a 25-kDa C_2H_2 zinc finger protein which specifically binds to two regions in the upstream activation sequence of the *STA1* gene, as judged by gel retardation and DNase I footprinting analyses. Disruption of the *NRG1* gene causes a fivefold increase in the level of the *STA1* transcript in the presence of glucose. The expression of *NRG1* itself is inhibited in the absence of glucose. DNA-bound LexA-Nrg1 represses transcription of a target gene 10.7-fold in a glucose-dependent manner, and this repression is abolished in both *ssn6* and *tup1* mutants. Two-hybrid and glutathione *S*-transferase pull-down experiments show an interaction of Nrg1 with Ssn6 both in vivo and in vitro. These findings indicate that Nrg1 acts as a DNA-binding repressor and mediates glucose repression of the *STA1* gene expression by recruiting the Ssn6-Tup1 complex.

In yeast, a large number of genes are turned off during growth on glucose (9, 37, 49). These glucose-repressible genes can be divided into three groups: (i) genes for metabolizing other carbon sources; (ii) genes encoding enzymes unique to gluconeogenesis; and (iii) genes involved in the Krebs cycle and in respiration. The Mig1 glucose repressor is a zinc finger protein and binds to the GC-rich motif identified in the promoters of several glucose-repressed genes, including the *GAL1*, *GAL4*, *SUC2*, and *MAL* genes (10, 13, 28, 29). In the absence of glucose, the Snf1 kinase inhibits the function of Mig1 protein directly or indirectly, leading to derepression of glucose-repressed genes (3, 4). Nuclear translocation of Mig1 is regulated by differential phosphorylation of the protein in response to glucose availability, and recruitment of the general repression complex Ssn6-Tup1 to the DNA-bound Mig1 is required for the repression (5, 17, 48). Disruption of the *MIG1* gene, however, only partially relieves glucose repression of *SUC2* and has little or no effect on glucose repression of other genes whose promoters contain the Mig1-binding sites (27, 31, 37, 50), indicating the involvement of other repressors in glucose repression. For instance, Mig2 was recently identified as a second repressor responsible for the remaining glucose repression of *SUC2* and contains zinc fingers very similar to those of Mig1 (24).

In *Saccharomyces cerevisiae* var. *diastaticus*, three unlinked homologous *STA* genes (*STA1*, *STA2*, and *STA3*) encode glucoamylase isozymes (GAI, GAII, and GAIII), which are responsible for enzymatic degradation of starch to glucose (16, 22, 25, 32, 35, 47, 52). Expression of the *STA* genes is regulated by complex interactions between positive and negative factors and their cognate elements (1, 19, 21, 33, 41). The negative regulation occurs at three different levels: (i) carbon catabolite repression by glucose (6, 34); (ii) repression by *STA10*, which is known as a repressor gene in *S. cerevisiae* (33); and (iii) diploid cell-specific repression (6, 34). The mechanisms underlying repression of the *STA* genes, however, are not yet understood.

In this study, to identify transcriptional regulators for glucose repression of the *STA1* gene, a plasmid was constructed by modifying the strategy used for cloning of the *MIG1* gene (29). The plasmid bears the *TPK2* gene, encoding a yeast cyclic AMP-dependent protein kinase, whose level of transcription is modulated by upstream regulatory elements (1, 41) of the *STA1* promoter. In derepressed conditions, cells with the *STA1p-TPK2* construct exhibit a slow-growth phenotype because of the toxic effects of the high level of Tpk2. Taking advantage of this phenotype, we isolated a gene, *NRG1*, whose presence in a multicopy vector confers normal growth to the cells in derepressed conditions. Here we report that the *NRG1* gene encodes a DNA-binding repressor required for the glucose repression of *STA1* gene expression.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study are listed in Table 1. Yeast transformations were done by a lithium acetate method (14). Genetic methods were performed as described elsewhere (40). *Escherichia coli* DH5α was used for propagation and selection of recombinant plasmids. To isolate multicopy suppressors of the *STA1* promoter, yeast cells were grown in solid synthetic medium containing 0.67% yeast nitrogen base without amino acids, 0.6% Casamino Acids, appropriate amino acids, and 2% Bacto Agar, supplemented with 3% soluble starch, 2% glycerol plus 2% ethanol, and 1% potassium acetate for induction (SC-SGE medium). For enzyme assay, yeast cells were grown at 30°C in synthetic minimal medium. Minimal medium containing Casamino Acids was composed of 0.67% yeast nitrogen base without amino acids plus appropriate amino acids. Carbon sources (2% glucose or 2% glycerol plus 2% ethanol) were added to minimal medium.

Isolation and analysis of the *NRG1* gene. The 2.46-kb *Nae*I-*Hin*dIII fragment containing the *TPK2* gene from plasmid pJN1 (a gift from H. Ronne) was subcloned into the *Stu*I-*Hin*dIII site of pUCSTA-G to construct plasmid pSTATPK2. pUCSTA-G contains upstream regulatory elements, TATA box, and portion of the open reading frame (ORF) of the *STA1* gene; the *Stu*I site is located between the TATA box and ATG codon. To construct a yeast integrative plasmid, the 3.2-kb *Kpn*I-*Stu*I fragment containing the *STA1* upstream activation sequence (UAS_{STA})-$TATA_{STA}$-*TPK2* fusion from pSTATPK2 was inserted into the *Kpn*I-*Bam*HI (blunt) of pASZ10 (45). The resulting plasmid (pAST2) was linearized by *Stu*I and used to transform strain AN20-1a. Stable transformants were selected in minimal medium without adenine and confirmed by genomic Southern analysis. The resulting strain (ST20-1a) was transformed with a yeast genomic library made in the multicopy vector pHR81 (a gift from H. Ronne). Transformants were plated on SC-SGE medium for induction of the *STA1*

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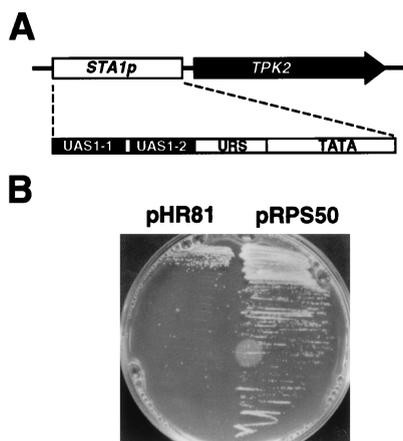
TABLE 1. Yeast strains used

Strains	Genotype	Reference
AN20-1a	*MAT*α *ura3 ade2 leu1 STA1 sta10*	1
AN20-5b	*MAT*α *ura3 leu1 STA1 sta10*	1
PHM20-5b	*MAT*α *ura3 leu1 nrg1Δ1::URA3 STA1 sta10*	This study
ST20-1a	*MAT*α *ura3 leu1 UAS_{STA}-TATA_{STA}-TPK2::ADE2 STA1 STA10*	This study
MCY829	*MAT*α *hisΔ200 lys2-801 ura3-52*	48
MCY1974	*MAT*α *hisΔ200 lys2-801 ura3-52 ade2-101 trp1Δ ssn6Δ9*	48
MCY2437	*MAT*α *hisΔ200 lys2-801 ura3-52 trp1Δ tup1Δ::TRP1*	48
PJ69-4A	*MAT*a *trp1 leu2 ura3 his3 gal4Δ gal80Δ GAL2-ADE2 lys::GAL1-HIS3 met::GAL7-lacZ*	15

promoter and incubated at 30°C for 7 days. Transformants growing faster than the host strain were selected, and plasmids were recovered from these transformants. As a second screening, strain AN20-5b was transformed with the recovered plasmids, and transformants with decreased glucoamylase production were isolated on synthetic medium containing 2% glycerol plus 2% ethanol. Plasmid pRPS50 was isolated as the multicopy suppressor in this genetic screen.

The 4.9-kb *Apa*I-*Bam*HI fragment of pRPS50 was subcloned into the *Apa*I-*Bam*HI site of pBlueScript-KS(+), generating pBSM1. The *Apa*I and *Bam*HI sites of pRPS50 originate from vector pHR81. The 3-kb *Spe*I (blunt)-*Hin*dIII fragment of pBSM1 was subcloned into *Xba*I (blunt)-*Hin*dIII site of pGEM7-Z, resulting in pGM1. The *Sma*I-*Eco*RI fragment containing the *URA3* gene from YEp24 was inserted into the *Sma*I-*Eco*RI site of pBlueScript-KS(+), generating pBSURA. The 1.1-kb *Xba*I-*Sal*I fragment containing the *NRG1* ORF of pGM1 was replaced by the 1.2-kb *Xba*I-*Sal*I fragment containing the *URA3* gene from pBSURA. The resulting plasmid was linearized by *Sph*I-*Bst*XI and transformed into strain AN20-5b. Transformants with the *nrg1Δ1::URA3* allele were isolated, and the disruption was confirmed by genomic Southern analysis.

LexA fusions. pLexA-Nrg1 was constructed by replacement of the *MIG1* gene with *NRG1* of plasmid pLexA-Mig1 (a gift from M. Carlson) (48). The *NRG1* ORF was amplified by using two oligonucleotides (5′-CGGGATCCCCATGTTTTACCCATATAAC-3′ and 5′-AGCTCGAGGATACCGTCAATTATTGTC-3′) and cloned into the *Bam*HI-*Sal*I site of pLexA-Mig1. The expression plasmid encoding only the LexA-binding domain (amino acids 1 to 87) was made by deleting the *MIG1* sequence from plasmid pLexA-Mig1. For transcriptional repression by LexA-Nrg1 fusion protein, plasmid JK1621 with four *lexA* operators (provided by M. Carlson) (48) and pLG669Z without *lexA* operators were used as reporter plasmids. β-Galactosidase activity was assayed in yeast cells permeabilized with chloroform and sodium dodecyl sulfate as described elsewhere (11).

Northern blot analysis. Cells were grown in synthetic medium containing 2% glucose or 2% glycerol plus 2% ethanol. Total RNA was isolated as described elsewhere (30), subjected to electrophoresis in a 1.2% agarose–formaldehyde gel, and then transferred onto a nitrocellulose membrane. To detect the *STA1* transcript, a 600-bp *Eco*RI-*Pvu*II internal fragment of the *STA1* gene was used as a probe (1). The *NRG1* transcript was detected by probing with a 700-bp *Bam*HI-*Xho*I fragment of plasmid pGEXNrg, constructed for expression of the glutathione *S*-transferase (GST)–Nrg1 fusion protein. A fragment of the *ACT1* gene was used as a control (8).

Gel retardation assay. The ORF of the *NRG1* gene was PCR amplified by using two oligonucleotides (5′-CGGGATCCATGTTTTACCCATATAAC-3′ and 5′-AGCTCGAGGATACCGTCAATTATTGTC-3′) and cloned into the *Bam*HI-*Xho*I site of pGEX4T-1, generating pGEXNrs. GST-Nrg1 was expressed in *E. coli* DH5α and purified essentially as described (43). Various amounts of GST-Nrg1 were incubated with a 5 ng of α-^{32}P-labeled probe (approximately 20,000 cpm) and 0.5 to 4 μg of poly(dI-dC) for 15 min at room temperature in a reaction volume of 20 μl containing 20 mM HEPES (pH 7.6), 1 mM MgCl₂, 60 mM KCl, 12% glycerol, 6 μg of bovine serum albumin, 10 μM ZnCl₂, and 1 mM dithiothreitol. The reaction samples were loaded onto 5% nondenaturing polyacrylamide gel and electrophoresed at 150 V for 2.5 h in a buffer of 22.5 mM Tris base, 22.5 mM boric acid, and 0.63 mM disodium EDTA, adjusted to pH 8.0. The gel was dried and autoradiographed. To confirm that Nrg1 protein is responsible for formation of the binding complex, GST-Nrg1 fusion protein was treated with thrombin, which cleaves specifically at the junction between GST and Nrg1. The amount of thrombin was determined empirically.

DNase I footprinting analysis. The coding strand of the 112-bp UAS1-1 was end labeled with [α-^{32}P]dATP by using Klenow enzyme. DNase I footprinting was performed as described elsewhere (42). For footprinting, a standard binding reaction mixture containing 0.5 μg of poly(dI-dC) was adjusted to 5 mM CaCl₂ and digested with empirically determined amounts of DNase I for 5 min. The reaction was stopped by adding EDTA and sodium dodecyl sulfate to final

concentrations of 25 mM and 0.1%, respectively, extracted once with phenol, precipitated with ethanol, and then analyzed on an 8% polyacrylamide gel containing 7 M urea. The gel was dried and exposed to X-ray film at −70°C. The size marker of the footprinting was prepared by G+A sequencing of UAS1-1 by the Maxam-Gilbert method.

Two-hybrid analysis. To identify Nrg1-interacting proteins, the Nrg1 fusion with the DNA-binding domain (DBD) of Gal4 (DBD-Nrg1) was made by ligating the *Bam*HI-*Xho*I fragment of the *NRG1* ORF from the GST-Nrg1 construct into *Bam*HI-*Sal*I of plasmid pGBDU (a gift from P. James) (15). A library of fusions between the activation domain (AD) of Gal4 and yeast genomic DNA (a gift from P. James) was transformed into strain PJ69-4A containing the DBD-Nrg1-expressing plasmid and plated on minimal medium lacking adenine and histidine, supplemented with 7 mM 3-aminotriazole. From 820,000 transformants, three positive clones were isolated. Plasmids were recovered from the three clones, and the genomic DNA of the plasmids was sequenced. All three plasmids contained regions of the N-terminal tetratricopeptide repeat domain of the *SSN6* gene.

In vivo interaction of Nrg1 with Ssn6 was tested with the plasmids expressing DBD-Nrg1 and AD-Ssn6 (Ssn6 residues 1 to 403 fused with the Gal4 AD) (48) and strain Y190 (12). As a positive control, plasmids expressing DBD-Snf1 and AD-Snf4 were used (7). β-Galactosidase activity was assayed as described elsewhere (38).

GST pull-down analysis. The *SSN6* ORF was cloned into the baculovirus transfer vector pBacPAK9 (Clontech) by PCR amplification of the gene with the oligonucleotides 5′-CGCGGATCCATGAATCCGGGCGGTGAACAAACA-3′ and 5′-TGCTCTAGATTAGTCGTCGTAGTTTTCATCTTC-3′. Recombinant baculoviruses were generated and used to infect *Spodoptera frugiperda* Sf21 insect cells. Insect cell extract was prepared as described previously (18). The Ssn6-containing extract (300 μg) was incubated with 10 μg of GST-Nrg1 for 3 h on ice. Ovalbumin was used as a control for nonspecific aggregation. Gluthione-agarose beads (30 μl) were added and incubated for 1 h at 4°C with constant agitation. Beads were precipitated and washed. Proteins in the pellet were eluted by being boiled in sample buffer and analyzed by Western blotting. Polyclonal rabbit anti-Ssn6 (36), polyclonal rabbit antiovalbumin (Sigma), and monoclonal mouse anti-GST (Santa Cruz) antibodies were used at 1:1,000 dilution. Horseradish peroxidase-conjugated anti-mouse (Pierce) and anti-rabbit (Amersham) secondary antibodies were used at 1:2,000 dilution. Detection was performed by enhanced chemiluminescence as instructed by the manufacturer (Pierce).

Glucoamylase assay. To measure glucoamylase activities, cells were grown at 30°C for 2.5 days and then pelleted by centrifugation. The culture supernatant was incubated in a total volume of 1 ml containing 100 mM sodium acetate (pH 5.2) and 1.62% soluble starch at 55°C for 30 min. Glucose produced by the action of glucoamylase on soluble starch was assayed by using a coupled glucose oxidase-peroxidase assay kit (Sigma). Amylase activity was determined as micrograms of glucose released per 100 μl of supernatant per A_{600} cells.

Nucleotide sequence accession number. The GenBank accession number of the *NRG1* gene is Z49812.

RESULTS

Isolation of a multicopy inhibitor of the *STA1* promoter. To investigate the negative regulation of *STA1* gene expression, we constructed a plasmid in which the *TPK2* gene, encoding a yeast cyclic AMP-dependent protein kinase, is transcribed from the *STA1* promoter containing upstream regulatory elements and TATA box of the *STA1* gene (1, 41) (Fig. 1A). Upon induction of the promoter by glycerol plus ethanol, starch, and 1% potassium acetate, cells harboring the plasmid exhibit a slow-growth phenotype (Fig. 1B), since overexpression of the kinase has toxic effects on cell growth (29). A yeast strain with a chromosomally integrated UAS_{STA}-TATA_{STA}-TPK2 fusion was transformed with a yeast genomic library made in a multicopy vector. To find plasmids that inhibit the *STA1* promoter function, we screened cells for the ability to grow normally on SC-SGE medium. Among 25,000 transformants, 57 colonies resumed normal growth. Plasmids were recovered from the 57 colonies and transformed into a Sta⁺ strain to measure glucoamylase activities. One plasmid, pRPS50, both conferred normal growth to the cells with integrated UAS_{STA}-TATA_{STA}-TPK2 (Fig. 1B) and significantly decreased glucoamylase production to the Sta⁺ strain (Table 2) under *STA1* promoter function-inducing conditions and was considered a candidate for the multicopy inhibitor of *STA1* promoter function.

The genomic DNA of pRPS50 was sequenced and found to

FIG. 1. Isolation of a multicopy inhibitor of the *STA1* promoter. (A) Schematic presentation of UAS$_{STA}$-TATA$_{STA}$-*TPK2* fusion. Subregions of UAS$_{STA}$ (UAS1-1, UAS1-2, and URS) are described by Ahn et al. (1). (B) ST20-1a cells were transformed with either pHR81 (vector control) or pRPS50, and transformants were grown at 30°C on SC-SGE medium.

FIG. 2. The amino acid sequence of Nrg1. (A) Predicted amino acid sequence of the *NRG1* coding region. The two zinc finger motifs are underlined. (B) Alignment of the Nrg1 zinc finger motifs to those of other zinc finger proteins. Numbers in parentheses refer to the number of the motif counted from the amino terminus of the protein. The two zinc fingers of yeast *MIG1* and *MSN2* genes are aligned. Also shown are the two fingers of Zif268 (Krox-24/Egr-1), Wilms' tumor protein (WT), Sp1, and the first two fingers of *Xenopus* transcription factor TFIIIA.

contain the ORF YDR043c, which has the capacity to encode a 231-amino-acid protein with a molecular weight of 25,000 (Fig. 2A). The gene was named *NRG1*. The predicted Nrg1 protein contains two tandem zinc finger motifs near the C terminus (Fig. 2A). The zinc finger regions are very similar to those of yeast Mig1 and Msn2 and are also closely related to those of mammalian early growth response proteins Egr-1/Zif268/Krox-24 and the Wilms' tumor locus protein (Fig. 2B).

Interestingly, the *NRG1* gene has previously been identified as *MSS1* in a genetic screen for a multicopy suppressor of elevated glucoamylase production caused by *sns1* mutation (1). The fact that the same gene was isolated by two different approaches to identify negative factors for *STA1* expression implies that the *NRG1* gene may play an important role in the negative regulation process.

Nrg1 is involved in glucose repression of *STA1* gene expression. Since overexpression of Nrg1 does not change the growth rate of the UAS$_{STA}$-TATA$_{STA}$-*TPK2*-integrated cells grown in glucose (data not shown), it is possible that Nrg1 mediates glucose repression of *STA1* gene expression. To test this idea, a strain with deletion of the entire *NRG1* coding region was generated. Deletion analysis revealed that *NRG1* is not essential for cell viability (data not shown). Glucoamylase production in wild-type and *nrg1*Δ cells was monitored during growth in minimal medium containing glucose as the sole carbon source. Under this repressed condition, wild-type cells produce negligible levels of glucoamylase activity. In contrast, *nrg1*Δ cells exhibit under the repressed condition dramatically increased glucoamylase activity comparable to the activity in a derepressed condition (Table 3), indicating that deletion of

Nrg1 relieves glucose repression of *STA1* gene expression.

Northern blot analysis was performed to determine whether the increased glucoamylase activity in *nrg1*Δ cells correlates with the transcription level of the *STA1* gene. The results show that the level of the *STA1* transcript under the repressed condition was five times higher in *nrg1*Δ cells than in wild-type cells, compared with band intensity of yeast actin transcript as an internal control (Fig. 3A). Interestingly, the level of the *NRG1* transcript was reduced about sixfold under the derepressed condition (Fig. 3B), indicating that the transcription of *NRG1* itself is regulated by different carbon sources. Taken together, the results of the glucoamylase assay and Northern analysis indicate that Nrg1 is required for glucose repression of *STA1* gene expression.

Nrg1 binds to an upstream element of the *STA1* promoter. The presence of zinc finger motifs in Nrg1 suggests that this protein may exert its role during glucose repression by binding to a specific DNA sequence of the *STA1* promoter. To examine this possibility, we purified recombinant Nrg1 as a fusion with GST and tested its binding to the upstream sequence elements (UAS1-1, UAS1-2, and an unidentified upstream repression

TABLE 2. Suppression of glucoamylase production by plasmid pRPS50[a]

Plasmid	Glucoamylase activity (U)[b]	Sta[+] phenotype
pHR81	14.5	++++
pRPS50	7.2	++

[a] Strain AN20-5b was transformed with either control plasmid pHR81 or pRPS50. Transformants were grown in synthetic medium lacking uracil and containing 2% glycerol plus 2% ethanol at 30°C.
[b] Average of at least duplicated experiments using three independent transformants with 10% deviation.

TABLE 3. Effects of *nrg1* disruption and carbon sources on *STA1* gene expression

Strains	Glucoamylase activity (U)[a]	
	Repressed	Derepressed
AN20-5b (wild type)	2.2	13.5
PHM20-5b (*nrg1Δ1*)[b]	10.8	12.6

[a] For the glucoamylase assay, cells were grown in synthetic medium containing 2% glucose (repressed condition) or 2% glycerol plus 2% ethanol (derepressed condition) at 30°C. Results are averages of at least duplicate experiments of three independent colonies with 10% deviation.
[b] Derivative of AN20-5b.
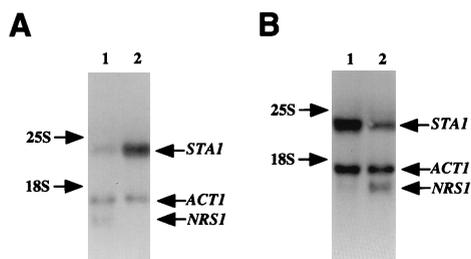
FIG. 3. *nrg1*Δ alleviates glucose repression of *STA1* transcription. (A) Effects of *nrg1*Δ on the *STA1* transcription under the repressed condition. Cells were cultured in synthetic medium containing 2% glucose as a carbon source, and then total RNA was prepared for Northern analysis. Lane 1, strain AN20-5b (*NRG1* [here designated *NRS1*]); lane 2, strain PHM20-5b (*nrg1*Δ). (B) Effects of carbon sources on the *NRG1* transcription. Strain AN20-5b (*NRG1*) was grown in synthetic medium containing either 2% glycerol plus 2% ethanol (derepressed condition; lane 1) or 2% glucose (repressed condition; lane 2), and then total RNA was extracted for Northern analysis. The yeast actin gene (*ACT1*) was used as an internal control.

sequence [URS]) located between nucleotides −493 and −893 of the *STA1* promoter. Gel retardation analysis revealed that GST-Nrg1 binds to UAS1-1 but not to UAS1-2 and URS (Fig. 4A). No binding activity was detected in reactions containing the GST control (Fig. 4A). The specificity of GST-Nrg1 binding to UAS1-1 was tested by competitive gel retardation experiments. Addition of a specific competitor, unlabeled UAS1-1, to the binding reactions reduced the intensity of the shifted band, while the addition of a nonspecific competitor, a DNA fragment of unrelated sequence at up to 64-fold molar excess or poly(dI-dC) at up to 12,000-fold molar excess, had little effect on the binding of GST-Nrg1 to UAS1-1 (Fig. 4B). To exclude the possibility that the DNA-protein complex was formed as a fortuitous consequence of the GST-Nrg1 recombinant protein, the junction between Nrg1 and GST of the purified GST-Nrg1 fusion was cleaved by using thrombin in solution. Gel retardation experiments with the thrombin-treated GST-Nrg1 and UAS1-1 resulted in a reduction in the size of the DNA-protein complex due to the smaller size of Nrg1 than of the GST-Nrg1 fusion protein (Fig. 4C), indicating that Nrg1 itself binds to UAS1-1. These results show that Nrg1 is a protein binding specifically to the UAS1-1 region of the *STA1* promoter.

Two regions in UAS1-1 are bound to Nrg1. DNase I footprinting analysis was performed with GST-Nrg1 to further characterize the Nrg1-binding regions in UAS1-1. The results show that two regions are protected by Nrg1 binding: one between nucleotides −864 and −874 (A box) and the other between nucleotides −851 and −862 (B box) (Fig. 5). Additionally, a DNase I-hypersensitive site appears concomitantly, as shown in Fig. 5. The same results were obtained from the experiments with thrombin-treated GST-Nrg1 (data not shown). The region from nucleotides −839 to −849 was protected with intact GST-Nrg1 but not with thrombin-treated GST-Nrg1, indicating that the protection in this region is not specific to Nrg1 binding. Although both Nrg1 and Mig1 function in glucose repression, analysis of the two regions protected by Nrg1 reveals no strong homology with the consensus Mig1-binding sequence, (G/C)(C/T)GG(G/A)G (23). Comparison of the Nrg1-binding sites with the consensus Mig1-binding sequence suggests that the nucleotide sequences CCCCT in A box and/or CCCTC in B box may be important for binding of Nrg1 to DNA (Fig. 5B).

LexA-Nrg1 represses transcription of a target gene. We assayed LexA-Nrg1, a Nrg1 fusion with the LexA DNA-bind-
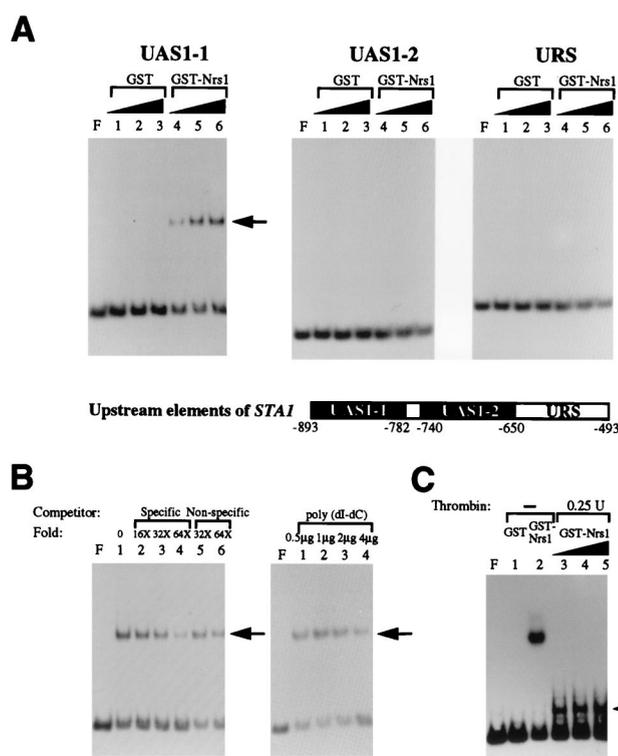
FIG. 4. Nrg1 binds to the UAS1-1 region of the *STA1* promoter. (A) Gel retardation assays were performed with GST-Nrg1 (here designated Nrs1) and labeled upstream elements of the *STA1* promoter (UAS1-1, UAS1-2, and unidentified URS). Lanes: F, no added protein; 1 to 3, reactions with increasing amounts of GST (0.05, 0.1, and 0.2 μg); 4 to 6, reactions with increasing amounts of GST-Nrg1 (0.05, 0.1, and 0.2 μg). In all the binding reactions, 0.5 μg of poly(dI-dC) was added. (B) Competitive gel retardation assays were performed with GST-Nrg1 and labeled UAS1-1. Lanes in left panel: F, no added protein; 1, no addition of competitor; 2 to 4, addition of increasing amounts of specific competitor (16-, 32-, and 64-fold molar excess of unlabeled UAS1-1); 5 and 6, addition of increasing amounts of nonspecific competitor (32- and 64-fold molar excess of pBluescript polylinker region). Lanes in right panel: F, no added protein; 1 to 4, addition of increasing amounts poly(dI-dC) (0.5, 1, 2, and 4 μg). (C) Gel retardation assays with thrombin-treated GST-Nrg1 and labeled UAS1-1. GST-Nrg1 was treated with 0.25 U of thrombin. Lanes: F, no added protein; 1, 0.2 μg of GST; 2, 0.2 μg of GST-Nrg1; 3 to 5, 0.1, 0.2, and 0.4 μg of thrombin-treated GST-Nrg1.

ing domain, for the ability to repress transcription of a target gene with *lexA* operators. Yeast cells with a reporter gene (*CYC1-lacZ*) containing either no or four *lexA* operators was transformed with a LexA-Nrg1 expression plasmid, and transformants were assayed for β-galactosidase activities under both the repressed (glucose) and derepressed (glycerol plus ethanol) conditions. Under the repressed condition, expression of the reporter gene containing LexA-binding sites was 10.7-fold lower than that of the reporter gene with no binding sites (Fig. 6), indicating that DNA-bound LexA-Nrg1 represses transcription. Consistent with previous results (48), LexA-Mig1 represses expression of the reporter gene 14-fold under the repressed condition (Fig. 6). Under the derepressed condition, neither LexA-Nrg1 nor LexA-Mig1 shows significant repression (Fig. 6).

Repression by LexA-Nrg1 requires Ssn6 and Tup1. Both LexA-Mig1 and LexA-Mig2 repress transcription in a Ssn6-Tup1-dependent manner (24, 48). To examine whether the repression by LexA-Nrg1 depends on Ssn6 and/or Tup1, repression in *ssn6*Δ and *tup1*Δ mutants was assayed under the
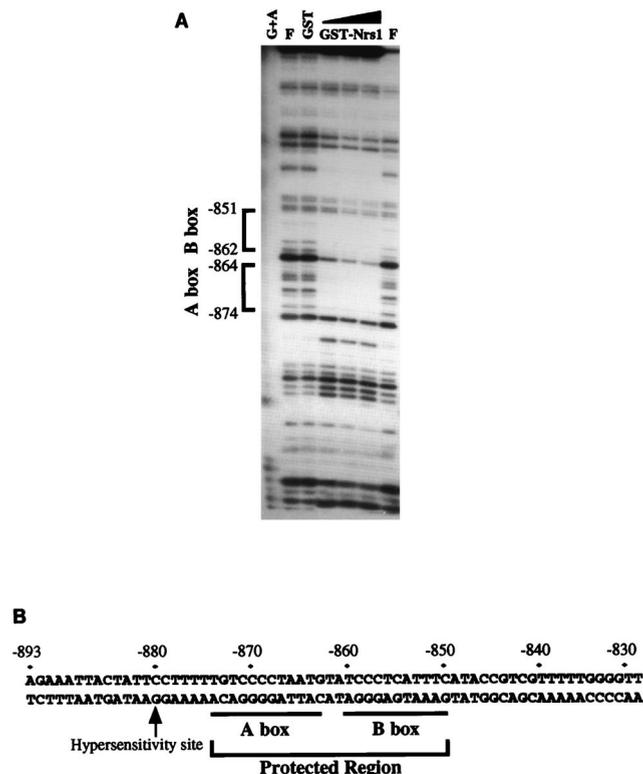
FIG. 5. Two regions in UAS1-1 are protected by Nrg1 binding. (A) DNase I footprinting assay of GST-Nrg1 (here designated Nrs1) on UAS1-1. The amount of DNase I was empirically determined. The assay was performed with either 0.2 μg of GST or increasing amounts of GST-Nrg1 (0.1, 0.2, and 0.4 μg). Lane F, no added protein. The size marker of the footprinting was prepared by G+A sequencing of UAS1-1 by the Maxam-Gilbert method. (B) Sequences of regions of UAS1-1 protected by Nrg1. Protected regions are underlined and named A box and B box. Also indicated is the DNase I hypersensitive site of UAS1-1.

Nrg1 interacts with Ssn6 both in vivo and in vitro. In a two-hybrid screening for detection of Nrg1-interacting proteins, we have identified regions of the N-terminal tetratricopeptide repeat domain of Ssn6 (data not shown). The interaction of Nrg1 with Ssn6 was confirmed by another two-hybrid assay (Fig. 7A). Cells with both DBD-Nrg1 and AD-Ssn6 (48) stimulated reporter gene expression more than 10-fold relative to control cells containing either DBD-Nrs1 or AD-Ssn6 alone. The level of the stimulation was comparable to that for a positive control for Snf1-Snf4 interaction (Fig. 7A).

These two-hybrid results, together with the genetic interaction between Nrg1 and Ssn6-Tup1, suggested the possibility of direct interaction between Nrg1 and Ssn6. GST pull-down experiments were performed to test the interaction. Insect cell extract containing overexpressed Ssn6 was incubated with purified GST-Nrg1. Proteins interacting with GST-Nrg1 were precipitated, and the pellet was analyzed. GST was used in a parallel reaction to control for specific interaction, and ovalbumin was added to each reaction mixture to control for nonspecific aggregation. The results revealed that Nrg1 interacts with Ssn6 in vitro (Fig. 7B). There was no detectable interaction between Nrg1 and Tup1 (data not shown). The results from the two-hybrid and GST pull-down analyses, therefore, indicate the physical interaction of Nrg1 with Ssn6 both in vivo and in vitro.

DISCUSSION

We provide here several lines of evidence for the identification of Nrg1 as a transcriptional repressor responsible for glucose repression of the *STA1* gene. First, *nrg1Δ* cells, when grown under the repressed conditions, exhibit dramatically increased glucoamylase activity which is comparable to that of cells grown under the derepressed conditions. Second, Northern analyses show that the increased glucoamylase level is correlated with the increased level of *STA1* transcript in *nrg1Δ* cells. Third, gel retardation and DNase I footprinting experiments demonstrate that Nrg1 binds specifically to UAS1-1 element of the *STA1* promoter. Fourth, tethering of Nrg1 to DNA via LexA-Nrg1 represses transcription of a target gene in glucose-grown cells, and the repression requires the Ssn6-Tup1 complex, which is needed for repression of diverse genes involved in many different cellular processes. And finally, two-

repressed condition. LexA-Nrg1 exhibited no significant repression of the reporter gene in both *ssn6Δ* and *tup1Δ* mutants (Fig. 6), indicating that repression by LexA-Nrg1 requires Ssn6 and Tup1.

			CYC1/LacZ Reporter		
Relevant Genotype	Expressed Proteins	Carbon Sources	0 —[UAS]→	4lexAop ●●●●—[UAS]→	Fold Repression
WT	LexA$_{1-87}$	glucose	355	166	2.2
	LexA-Nrs1		437	41	10.7
	LexA-Mig1		475	35	14.2
WT	LexA$_{1-87}$	glycerol ethanol	1495	545	2.7
	LexA-Nrs1		2036	670	3.0
	LexA-Mig1		1381	657	2.1
ssn6Δ	LexA$_{1-87}$	glucose	101	40	2.2
	LexA-Nrs1		113	44	2.5
tup1Δ	LexA$_{1-87}$	glucose	55	24	2.3
	LexA-Nrs1		58	26	2.2

FIG. 6. Transcriptional repression by LexA-Nrg1. The *CYC1-lacZ* reporters in this experiment were pLG669Z (no *lexA* operator) and JK1621 (four *lexA* operators) (48). LexA1-87 DNA-binding domain, LexA-Nrg1 (here designated Nrs1), or LexA-Mig1 was expressed in either wild-type (wt) or mutant strains. Cells were grown in synthetic media lacking uracil and histidine and containing either 2% glucose (repressed condition) or 2% glycerol plus 2% ethanol (derepressed condition). β-Galactosidase activity values are averages of duplicated experiments using three independent transformants.
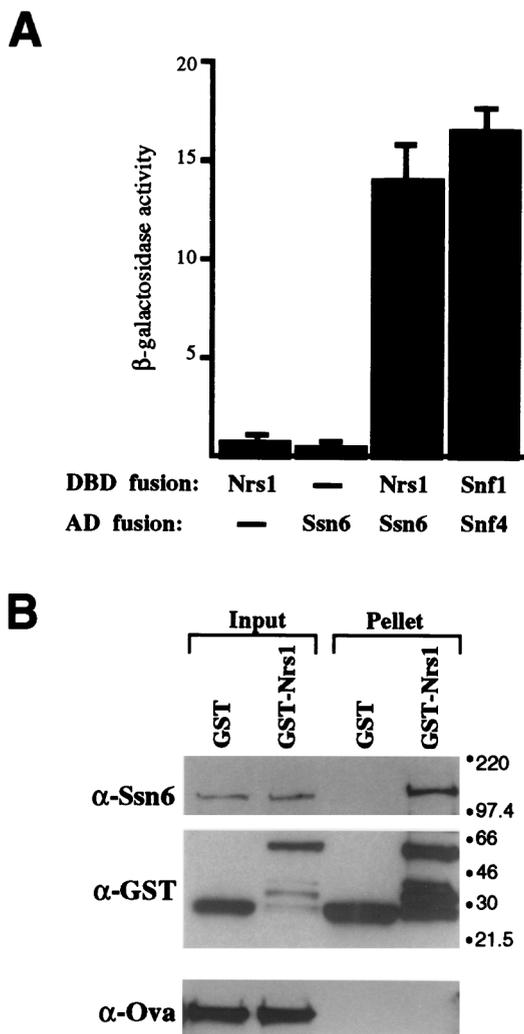
A

B

FIG. 7. Nrg1 interacts with Ssn6. (A) In vivo interaction. Plasmids expressing DBD-Nrg1 (here designated Nrs1) and AD-Ssn6 fusions were transformed into strain Y190 and assayed for β-galactosidase activity. As a positive control, plasmids expressing DBD-Snf1 and AD-Snf4 were transformed. Negative controls included cells with Gal4 DBD and AD-Ssn6 and cells with DBD-Nrg1 and Gal4 AD. β-Galactosidase activity values are averages of duplicated experiments using three independent transformants. (B) In vitro interaction. Ssn6-containing insect cell extract was incubated with GST or GST-Nrg1, and the GST proteins and their interacting proteins were precipitated by using glutathione-agarose beads. Ovalbumin (Ova) was added to each reaction mixture to serve as a control for specific precipitation. Fractions of input (1/30) and pellet (1/3) were analyzed by Western blotting with specific antibodies. Sizes are indicated in kilodaltons.

hybrid and GST pull-down experiments demonstrate the physical interaction between Nrg1 and Ssn6 both in vivo and in vitro.

Nrg1 joins Mig1 and Mig2 as a DNA-binding repressor for glucose repression. The putative Nrg1-binding sequence, CCCCT and/or CCCTC, is, however, quite different from the consensus Mig1-binding sequence, (G/C)(C/T)GG(G/A)G, and other known consensus sequences for zinc finger proteins. Mig1 and Mig2 function as repressors of various genes such as *GAL1*, *GAL4*, *SUC2*, and *MAL* and bind to the same DNA sequence with different affinities (10, 13, 24, 28, 29). Analysis of the promoter regions of the Mig1/2-dependent glucose-repressible genes reveals no Nrg1-binding sequence motif, implying that the main targets of the Nrg1 function are *STA*

genes. Although we cannot exclude the possibility of the involvement of Mig1, Mig2, and/or some unknown repressors, we suggest that Nrg1 is the major repressor responsible for the glucose repression of the *STA* genes since *nrg1Δ* cells almost completely alleviate the glucose repression. Consistent with this notion are our observations that the *STA* promoters do not contain a consensus Mig1-binding sequence and *mig1Δ* cells do not exhibit increased glucoamylase activity under repressed conditions (our unpublished data). It was also reported that Mig1 is dispensable for glucose repression of *STA2* (16). Establishment of a consensus Nrg1-binding sequence, however, awaits more exhaustive investigation; more importantly, consideration should also be given to effects of promoter context since the total output of transcription of a given gene is a consequence of a complicated interaction of both positive and negative regulators. For example, not all genes containing the Mig1-binding site are affected by *mig1* deletion (26, 37), and in some promoters more than one repressor is required for complete glucose repression (24, 31).

The binding of Nrg1 to UAS1-1 is consistent with the previous finding that the UAS1 region confers glucose repression when fused to a reporter gene (1). The affinity of Nrg1 for UAS1-1, however, seems to be weak compared with that of Mig1 for *SUC2*, *GAL1*, and *GAL4* (10, 28, 29), since the addition of a specific competitor in 64-fold molar excess to the binding reactions does not completely abolish the Nrg1-DNA complex (Fig. 5B). This weak binding of Nrs1 may explain why glucose repression of the *STA1* genes is not as tight as that of *GAL1* or *SUC2*; Sta⁺ strains synthesize significant amounts of glucoamylase in rich medium containing glucose, albeit at levels lower than those of the same strains grown in rich medium containing glycerol plus ethanol (34).

Our genetic and biochemical evidence indicates that glucose repression by Nrg1 involves recruitment of the Ssn6-Tup1 complex. The general corepressor Ssn6-Tup1 is required for regulation of a subset of genes that is involved in many cellular processes, including cell type specificity, meiosis, oxygen utilization, and sugar utilization (2, 20, 46, 51), and most DNA-binding repressors involved in these processes, e.g., Mig1, Rox1, and α2, interact with the tetratricopeptide domain of Ssn6 (2, 44, 48). The Nrg1-Ssn6 interaction as well is mediated through the tetratricopeptide domain. Though comparison of the primary amino acid sequence of Nrg1 with those of Mig1, Mig2, Rox1, and α2 reveals no significant homology, it would be interesting to determine the structural requirement for binding of the proteins to the tetratricopeptide domain of Ssn6.

How does the glucose repression of *STA1* occur? Based on our observation that the transcription of *NRG1* is induced by glucose, we suggest a model in which Nrg1, in the presence of glucose, is highly produced and binds to upstream sequence of the *STA1* gene to recruit Ssn6-Tup1 for repression of *STA1* expression. In the absence of glucose, the expression level of *NRG1* is decreased and hence *STA1* expression is derepressed. This repression mechanism of Nrg1 is different from that of Mig1, the function of which is regulated by nuclear translocation through changes in its phosphorylation status (5). How is the expression of *NRG1* regulated? One possibility is that *NRG1*, like *HXT* genes, is induced in the presence of glucose (31). A possible candidate responsible for the induction of *NRG1* is the *SNS1* gene product. Previously, *NRG1* has been identified as a multicopy suppressor of *sns1* mutation which elevates glucoamylase production (1), and our recent data show that the *sns1* mutation decreases the expression of the *NRG1* gene (unpublished data).

In this report, we suggest that Nrg1 is a key element for

glucose repression of the *STA1* gene as a DNA-binding repressor. Further genetic and biochemical analysis of Nrg1 together with other regulatory factors should reveal the molecular mechanism for the negative regulations of the *STA* genes.

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