MOLECULAR AND CELLULAR BIOLOGY, Sept. 2004, p. 7695–7706 Vol. 24, No. 17
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.17.7695–7706.2004

Glucose Repression of *STA1* Expression Is Mediated by the Nrg1 and Sfl1 Repressors and the Srb8-11 Complex

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Received 12 December 2003/Returned for modification 2 February 2004/Accepted 13 May 2004

In the yeast *Saccharomyces diastaticus*, expression of the *STA1* gene, which encodes an extracellular glucoamylase, is negatively regulated by glucose. Here we demonstrate that glucose-dependent repression of *STA1* expression is imposed by both Sfl1 and Nrg1, which serve as direct transcriptional repressors. We show that Nrg1 acts only on UAS1, and Sfl1 acts only on UAS2, in the *STA1* promoter. When bound to its specific site, Sfl1 (but not Nrg1) prevents the binding to UAS2 of two transcriptional activators, Ste12 and Tec1, required for *STA1* expression. We also found that Sfl1 contributes to *STA1* repression by binding to the promoter and inhibiting the expression of *FLO8*, a gene that encodes a third transcriptional activator involved in *STA1* expression. In addition, we show that the levels of Nrg1 and Sfl1 increase in glucose-grown cells, suggesting that the effects of glucose are mediated, at least in part, through an increase in the abundance of these repressors. *NRG1* and *SFL1* expression requires the Srb8-11 complex, and correspondingly, the Srb8-11 complex is also necessary for *STA1* repression. However, our evidence indicates that the Srb8-11 complex does not associate with either the *SFL1* or the *NRG1* promoter and thus plays an indirect role in activating *NRG1* and *SFL1* expression.

In yeast, glucose negatively regulates the expression of genes involved in the metabolism of alternative carbon sources. This phenomenon, known as glucose repression, involves complex interactions between DNA-binding repressors, their cognate elements, and components of the transcriptional machinery. Several zinc finger proteins, such as Mig1, Mig2, Nrg1, and Nrg2, repress transcription of the *SUC2*, *GAL1*, *MAL*, *FLO11*, and *STA1* genes by binding to specific elements and recruiting the general corepressor Ssn6-Tup1 to the promoters of the target genes (8, 14, 19, 20, 23, 26, 27, 31, 38).

In the yeast *Saccharomyces diastaticus*, three unlinked homologous *STA* genes (*STA1*, *STA2*, and *STA3*) encode glucoamylase isozymes (GAI, GAII, and GAIII) that degrade starch to glucose. The expression of *STA1* is repressed at three different levels: (i) glucose repression (9, 33), (ii) repression by STA10 (32), and (iii) diploid cell-specific repression (9, 33). It has been reported that STA10 repression is due to a mutation in the activator *FLO8*, which is critical for *STA1* expression (12, 21). In glucose repression, Nrg1 is thought to bind directly to UAS1-1 of the *STA1* promoter and to recruit the Ssn6-Tup1 corepressor (31).

The *STA1* promoter is almost identical to the *FLO11* promoter, which encodes a mucin-like cell surface glycoprotein essential for pseudohyphal differentiation, invasive growth, and flocculation (12). *STA1* and *FLO11* are coregulated in response to various environmental signals, and their expression is controlled in a complicated manner by several transcriptional activators, e.g., Flo8, Mss11, Ste12, and Tec1 (11–13, 30, 35), and transcriptional repressors (23, 30, 31, 34). Glucose depletion causes derepression of *FLO11* expression in haploid cells, whereas nitrogen starvation causes derepression in diploid cells (6, 23, 29, 40). The 5′ upstream region of *FLO11* contains an Nrg1 binding site, and transcription is repressed by Nrg1 and Nrg2 as well as by Sfl1 (23, 30, 34). However, it is not clear whether Sfl1 is also involved in glucose repression of *STA1* expression.

Sfl1 represses the transcription of several genes, including *SUC2* and *FLO11*, and interacts physically and functionally with Srb and other mediator proteins to repress the transcription of target genes. DNA-bound LexA-Sfl1 represses the transcription of a reporter gene, and Ssn6-Tup1 is required for Sfl1-mediated repression (5, 30, 37). Sfl1 forms multimers via a coiled-coil domain, and this multimerization is thought to be important for binding to DNA. Tpk2, a catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase A (PKA), inhibits multimerization and Sfl1 binding to DNA (30).

Srb proteins, such as Srb8, -9, -10, and -11, form a large complex and play an important role in the activation and repression of gene expression (2). The Srb8-11 complex is also somewhat involved in transcriptional repression by DNA-bound LexA-Ssn6 and LexA-Tup1 (22, 24, 25). The purified complex phosphorylates the C-terminal domain of RNA polymerase II on serines 2 and 5 prior to initiating the formation of a complex on the target promoter (2, 17). The complex is also involved in the transcription of several genes, including *GAL1* and gluconeogenic genes, by positively regulating gene-specific activators (22, 39). Moreover, this complex phosphorylates transcriptional activators, such as Ste12, Sip4, Gal4, Msn2, and Gcn4, and regulates their activity. It also promotes the degradation of Ste12 and Gcn4 and is important for activation by Sip4 (3, 18, 28, 39).

In this study, we have used a UAS_{STA1}-$CYC1_{TATA}$-*lacZ* expression system to examine the roles of different regions of the *STA1* promoter in *STA1* transcription, and we have isolated

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TABLE 1. *S. diastaticus* strains used in this study

Strain	Genotype	Reference or source
SPX15-3D	*MAT***a** *STA1 leu1 thr1 FLO8*	32
YPH499	*MAT*α *ade2 his3 leu2 thr1 lys2 trp1 ura3 flo8*-1	35
KHS 182	*MAT*α *STA1 leu2 his3 trp1 ura3 FLO8*	21
KHS 182-11	*MAT*α *STA1 leu2 his3 trp1 ura3 nrg1*Δ::*TRP1*	This study
KHS 182-12	*MAT*α *STA1 leu2 his3 trp1 ura3 sfl1*Δ::*TRP1*	This study
KHS 182-13	*MAT*α *STA1 leu2 his3 trp1 ura3 nrg1* Δ::*HIS3 sfl1*Δ::*TRP1*	This study
KHS 182-14	*MAT*α *STA1 leu2 his3 trp1 ura3 ssn6*Δ::*URA3*	This study
KHS 182-15	*MAT*α *STA1 leu2 his3 trp1 ura3 sin4*Δ::*TRP1*	This study
KHS 182-16	*MAT*α *STA1 leu2 his3 trp1 ura3 srb8*Δ::*TRP1*	This study
KHS 182-17	*MAT*α *STA1 leu2 his3 trp1 ura3 srb9*Δ::*TRP1*	This study
KHS 182-18	*MAT*α *STA1 leu2 his3 trp1 ura3 srb10*Δ::*TRP1*	This study
KHS 182-19	*MAT*α *STA1 leu2 his3 trp1 ura3 sfl1*Δ::*URA3 srb10*Δ::*TRP1*	This study
KHS 182-20	*MAT*α *STA1 leu2 his3 trp1 ura3 nrg1*Δ::*HIS3 srb10*Δ::*TRP1*	This study
KHS 182-21	*MAT*α *STA1 leu2 his3 trp1 ura3 srb11*Δ::*TRP1*	This study
KHS 182-22	*MAT*α *STA1 leu2 his3 trp1 ura3 sfl1*Δ::*URA3 srb11*Δ::*TRP1*	This study
KHS 182-23	*MAT*α *STA1 leu2 his3 trp1 ura3 nrg1*Δ::*HIS3 srb11*Δ::*TRP1*	This study
KHS 182-30	*MAT*α *STA1 leu2 his3 trp1 ura3 FLO8-HA*::*LEU2*	This study
KHS 182-31	*MAT*α *STA1 leu2 his3 trp1 ura3 MSS11-HA*::*LEU2*	This study
KHS 182-32	*MAT*α *STA1 leu2 his3 trp1 ura3 STA12-HA*::*LEU2*	This study
KHS 182-33	*MAT*α *STA1 leu2 his3 trp1 ura3 TEC1-HA*::*LEU2*	This study
KHS 182-40	*MAT*α *STA1 leu2 his3 trp1 ura3 SRB10-HA*::*LEU2*	This study
KHS 182-40-1	*MAT*α *STA1 leu2 his3 trp1 ura3 nrg1*Δ::*TRP1 SRB10-HA*::*LEU2*	This study
KHS 182-40-2	*MAT*α *STA1 leu2 his3 trp1 ura3 sfl1*Δ::*TRP1 SRB10-HA*::*LEU2*	This study
KHS 182-40-3	*MAT*α *STA1 leu2 his3 trp1 ura3 nrg1*Δ::*HIS3 sfl1*Δ::*TRP1 SRB10-HA*::*LEU2*	This study

SFL1 as a multicopy inhibitor of the *STA1* promoter. We found that two of the upstream elements of the *STA1* promoter, UAS1-1 and UAS2-2, mediate glucose repression of *STA1* expression and are the targets of Nrg1 and Sfl1, respectively. We also provide evidence that Sfl1 competes with Ste12 and Tec1 for binding of the UAS2 region of the *STA1* promoter and represses *FLO8* expression. In glucose repression, Nrg1 and Sfl1 inhibit *STA1* expression through different mechanisms. Furthermore, we show that the Srb8-11 complex plays critical roles in glucose repression of *STA1* expression and that this complex indirectly activates *NRG1* and *SFL1* expression. Finally, we suggest that increased levels of the repressors Nrg1 and Sfl1 are important in mediating glucose repression of *STA1* expression.

MATERIALS AND METHODS

Strains and media. The *S. diastaticus* strains used in this work are listed in Table 1. KHS 182 was constructed by mating YHP499 (*MAT*α *ade2 his3 leu2 lys2 trp1 ura3 flo8-1*) with SPX15-3D(*MAT***a** *leu1 thr1 STA1 FLO8*). Yeast cells were grown at 30°C in YPD (1% yeast extract, 2% tryptone, and 2% glucose) or a synthetic medium containing 0.67% yeast nitrogen base supplemented with appropriate amino acids and carbon sources (2% glucose or 2% glycerol-ethanol). Mutant strains were constructed by replacing the open reading frames (ORFs) with *TRP1, HIS3*, or *URA3* by PCR-mediated disruption, and mutations were confirmed by PCR. To construct the hemagglutinin (HA)-tagged strains, pRS305-*FLO8*-HA, pRS305-*MSS11*-HA, pRS305-*STE12*-HA, pRS305-*TEC1*-HA, and pRS305-*SRB10*-HA were linearized with BglII, SphI, NcoI, NheI, and SphI, respectively, and the linearized DNA fragments were integrated into their respective genomic loci. Tagged strains were confirmed by PCR analysis, glucoamylase assay, and Western blot analysis with an anti-HA (α-HA) antibody.

Plasmids. The plasmids used in this study are listed in Table 2. To construct the pRS-HA plasmids, the triple-HA tag was amplified by using primers that create terminal SalI/XhoI sites, and the resulting HA fragment was subcloned into SalI/XhoI sites in pRS305 and pRS325. To construct pRS305-ORF-HA, the entire *FLO8*, *MSS11, STE12*, *TEC1*, and *SRB10* ORFs were amplified by PCR using primers that create terminal BamHI/XhoI (for *FLO8*) and BamHI/SalI (for the other ORFs) sites and then subcloned into pRS305-HA at the corresponding sites. pLG-UAS2a containing a mutated Sfl1 binding site (CGCA) was constructed by PCR using *Pfu* polymerase (Stratagene) and the mutagenic primer pairs 5′-GGTTTTTTCTTCTGTTTCTTGACACGCAAAT GTTG CCC AAAGAGTTTCG-3′ and 5′-CGAAACTCTTTGGGCAACATTTGCGTGTC AAGAAACAGAAGAAAAAACC-3′. The construct generated was confirmed by sequencing.

Cloning of *SFL1*. To isolate a multicopy inhibitor(s) that acts on the UAS2 of the *STA1* promoter, pLG-UAS2A, which bears a BglII linker at the SmaI site of pLG-UAS2, was used to generate a genomic library. Yeast genomic DNA isolated from KHS 182 was partially digested with Sau3AI, and fragments with an average size of 5 to 10 kb were collected by sucrose velocity sedimentation. The resulting fragments were inserted at the BglII site of pLG-UAS2A. The genomic library was transformed into KHS 182, and the resulting transformants were incubated on BMM–X-Gal plates (1). Under these conditions, transformants containing the control pLG-UAS2 plasmid form blue colonies, whereas cells bearing a repressor acting on UAS2 would generate white colonies. On this basis, we isolated 3 white colonies from about 100,000 transformants on BMM–X-Gal plates. Plasmids were recovered from these three candidates, B42, C13, and C33, and retransformed into KHS 182 to check their effect. Transformants harboring the control plasmid, pLG-UAS2, had high glucoamylase and β-galactosidase activities, whereas transformants bearing plasmid B42, C13, or C33 all had reduced levels of both activities (data not shown).

Glucoamylase assay, Northern blot analysis, and β-galactosidase assay. Glucoamylase assays and Northern blot analyses were performed as described previously (31). β-Galactosidase was assayed as described previously (1).

GST pull-down assay. Total extracts (1 mg) prepared from the integrated *SRB10*-HA strain were incubated for 3 h on ice with 5 μg of purified glutathione *S*-transferase (GST)-fused proteins. Glutathione-agarose beads (25 μl) were added and incubated for 2 h at 4°C with constant agitation. The beads were pelleted and washed four times. Proteins in the pellet were eluted by boiling the beads in sample buffer and were analyzed by Western blotting with monoclonal α-HA antibodies (Santa Cruz).

Preparation of protein extracts and immunoblot analysis. Total proteins were extracted as described previously (42). The extraction buffer was 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, and 10% glycerol, containing 2 mM phenylmethylsulfonyl fluoride and Complete protease inhibitor cocktail (Sigma). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 8 to 10% acrylamide) and analyzed by immunoblotting with α-HA (Santa Cruz) and α-actin (Sigma) antibodies.

TABLE 2. Plasmids used in this study

Plasmid	Description	Reference or source
pRS305	*LEU2*	36
pRS305-HA	Triple HA tags in pRS305 (SalI/XhoI)	This study
pRS325	2μm *LEU2*	4
pRS323	2μm *HIS3*	4
pRS325-HA	Triple HA tags in pRS325 (SalI/XhoI)	This study
pRS325-*ADH1*p-HA	*ADH1* promoter (−1500 to −1) in pRS325-HA (SacI/NotI)	This study
pRS325-*lacZ*	*lacZ* in pRS325 (BamHI)	This study
pLG 669-Z	2μm *URA3* UAS_{CYC1}-*CYC1-lacZ*	15
pLG 670-Z	2μm *UAS3 CYCI-lacZ*	15
JK1621	2μm *URA3* 4*lexA*op-UAS_{CYCI}-*CYC1*-lacZ	20
pLG-STA1-1	−2105 to −1 region of the *STA1* promoter in pLG-670Z	This study
pLG-STA1-2	−2105 to −882 region of the *STA1* promoter in pLG-670Z	This study
pLG-STA1-3	−1642 to −882 region of the *STA1* promoter in pLG-670Z	This study
pLG-STA1-4	−1642 to −1380 region of the *STA1* promoter in pLG-670Z	This study
pLG-UAS1	−2105 to −1642 region of the *STA1* promoter in pLG-670Z	This study
pLG-UAS1-1	−2105 to −1905 region of the *STA1* promoter in pLG-670Z	This study
pLG-UAS1-2	−1905 to −1642 region of the *STA1* promoter in pLG-670Z	This study
pLG-UAS2	−1380 to −882 region of the *STA1* promoter in pLG-670Z	This study
pLG-UAS2-1	−1380 to −1147 region of the *STA1* promoter in pLG-670Z	This study
pLG-UAS2-2	−1147 to −882 region of the *STA1* promoter in pLG-670Z	This study
pLG-UAS2A	BglII linker in pLG-UAS2 (SmaI)	This study
pLG-UAS2a	Replacement of AGAA with CGCA in pLG-UAS2	This study
pLG-UAS1-*NRGI*	2.0-kb fragment containing *NRG1* ORF in pLG-UAS1	This study
pLG-UAS2-*SFL1*	3.5-kb fragment containing *SFL1* ORF in pLG-UAS2	This study
pLG-UAS2a-*SFL1*	3.5-kb fragment containing *SFL1* ORF in pLG-UAS2a	This study
pRS323-*NRG1*	2.0-kb-fragment containing *NRG1* ORF in pRS323	This study
pRS323-*SFL1*	3.5-kb fragment containing *SFL1* ORF in pRS323	This study
pRS325-*NRG1*-HA	2.0-kb fragment containing *NRG1* ORF in pRS325-HA	This study
pRS325-*SFL1*-HA	3.5-kb fragment containing *SFL1* ORF in pRS325-HA	This study
pRS325-*ADH1*p-*NRGI*-HA	*NRGI* ORF in pRS325-*ADHI*p-HA	This study
pRS325-*ADH1*p-*SFL1*-HA	*SFL1* ORF in pRS325-*ADH1*p-HA	This study
p*NRG1-lacZ*	*NRG1* promoter (bp − 1200 to −1) in pRS325-*lacZ* (NotI)	This study
p*SFL1-lacZ*	*SFL1* promoter (bp −1500 to −1) in pRS325-*lacZ* (NotI)	This study
pLexA-*NRG1*	*NRG1* in pSH2-1 (BamHI/XhoI)	31
pLexA-*SFL1*	*SFL1* in pSH2-1 (BamHI/XhoI)	This study
pRS305-*FLO8*-HA	*FLO8* in pRS305-HA (BamHI/SalI)	This study
pRS305-*MSS11*-HA	*MSS11* in pRS305-HA (BamHI/XhoI)	This study
pRS305-*STE12*-HA	*STE12* in pRS305-HA (BamHI/XhoI)	This study
pRS305-*TEC1*-HA	*TEC1* in pRS305-HA (BamHI/XhoI)	This study
pRS305-*SRB10*-HA	*SRB10* in pRS305-HA (BamHI/XhoI)	This study

ChIP assays. To detect protein-DNA interaction, chromatin immunoprecipitation (ChIP) assays were performed as described by Hecht et al. (16) with minor modifications. Cells were grown in a 2% glucose or 2% glycerol-ethanol medium to an optical density at 600 nm (OD_{600}) of 1.0 and were treated with formaldehyde (1%) to cross-link DNA and proteins. Extracts were sonicated, and equal amounts of extract were incubated with an α-HA antibody at 4°C overnight. GammaBind G Sepharose beads (Amersham) were added to precipitate DNA-HA-tagged proteins, and the beads were washed four times. Elution buffer was added and incubated at 65°C overnight. DNA fragments were purified with a QiaQuick PCR column (QIAGEN), and the immunoprecipitated DNAs were amplified by 30 cycles of PCR to detect the upstream regions of the *STA1* promoter in the pLG vector series and the endogenous *STA1* promoter by using the following primer pairs: for CYC1, 5′-GAAAGGAAAGCAGGAAAGG-3′ and 5′-TATACACGCCTGGCGGATCTG-3′; for UAS1-2, 5′-CCTATTCTCA TCGAGAGCCGAG-3′ and 5′-CAAGTACTGCAGTGCATGTCC-3′); for UAS2-1, 5′-GGTAAGATTTGTTCTATG-3′ and 5′-GAACTTTCCAGGCTCA CC-3′; for UAS2-2, 5′-GGTGTGCCTGGAAAGTTC-3′ and 5′-GAGCAATC AGCAGTTCTTTG-3′; and for TATA, 5′-CTTAACAAATATGTTCAAGC-3′ and 5′-TGGATTTTTGAGGCCTACC-3′. The CYC1 primer pairs were used to detect the upstream activation sequence (UAS) of the *STA1* promoter within the pLG series. The PCR products were separated by 2% agarose gel electrophoresis and photographed.

RESULTS

Sfl1 and Nrg1 are transcriptional repressors for glucose repression of *STA1* expression. It was previously reported that Nrg1 is a transcriptional repressor that acts on the UAS1 region (−2105 to −1642) of the *STA1* promoter and mediates glucose repression of *STA1* expression (31). However, *nrg1*Δ did not greatly relieve glucose repression of *STA1* in the wild-type strain KHS 182 (Fig. 1B), suggesting that some additional transcriptional repressor(s) exists to repress *STA1* expression. To explore this possibility, we tested whether another upstream region besides UAS1 is also involved in glucose repression. To identify the UAS(s), we determined the β-galactosidase activities of plasmid-based UAS_{STA1}-$CYC1_{TATA}$-*lacZ* reporter constructs. As shown in Fig. 1A, not only UAS1 (−2105 to −1642) but also the 498-bp fragment (−1380 to −882) referred to as UAS2 causes strong expression of the reporter gene under derepressed conditions (2% glycerol-ethanol) but complete repression under repressed conditions (2% glucose). This result indicates that UAS2

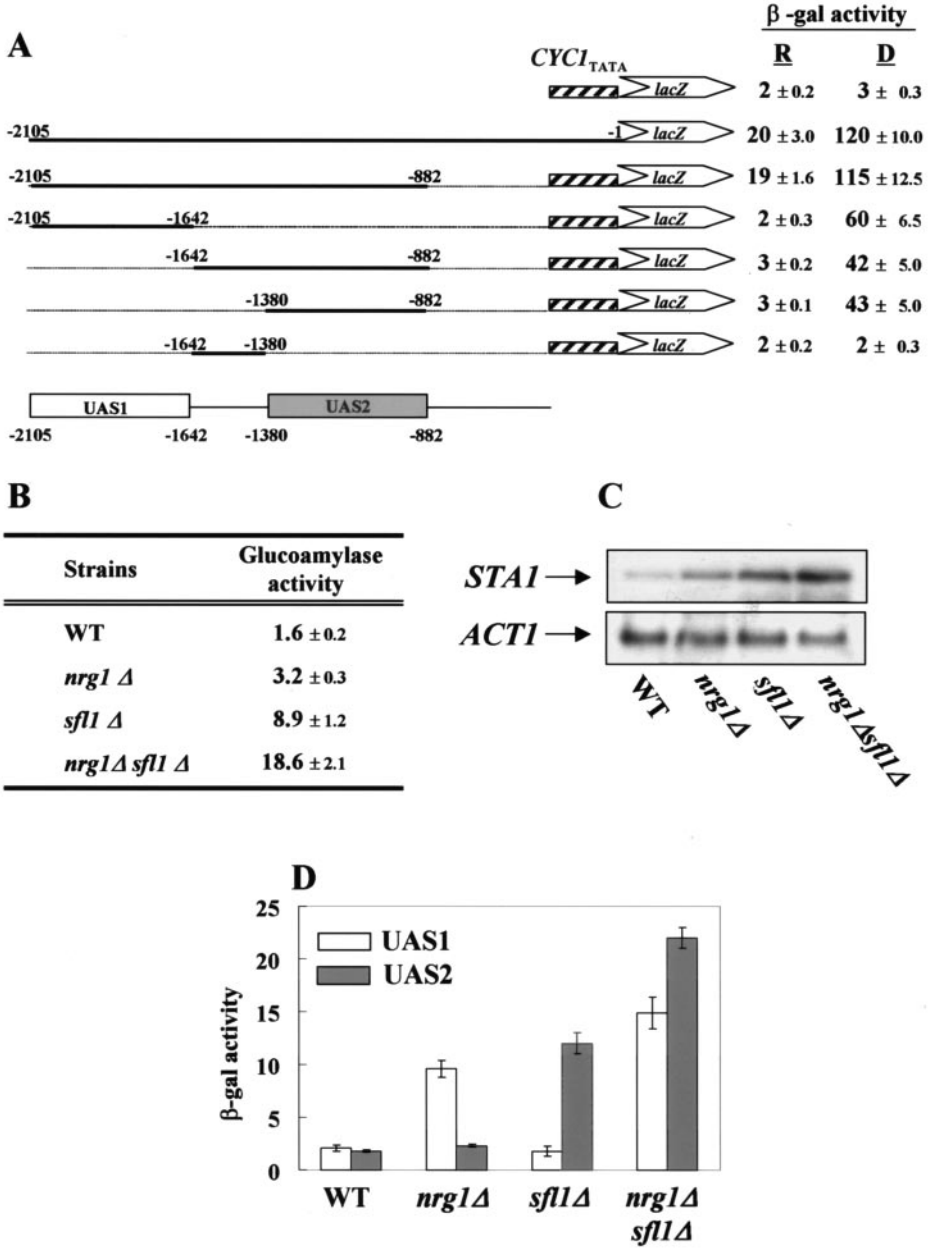

FIG. 1. Nrg1 and Sfl1 act on UAS1 and UAS2, respectively. (A) DNA fragments carrying the *STA1* promoter were inserted into pLG 670-Z containing a $CYC1_{TATA}$-*lacZ* reporter gene, yielding the pLG-UAS series. These vectors were transformed separately into KHS 182. Three independent colonies obtained with each plasmid were tested for β-galactosidase activity under repressed (2% glucose) (R) or derepressed (2% glycerol-ethanol) (D) conditions. (B) Wild-type (WT) and mutant strains were grown in synthetic medium containing 2% glucose as a carbon source. Glucoamylase activities are averages from three independent experiments. (C) Total RNA was prepared from the same strains for Northern blot analysis. The yeast actin gene (*ACT1*) was used as an internal control. (D) pLG-UAS1 or pLG-UAS2 was introduced into wild-type, *nrg1*Δ, *sfl1*Δ, or *nrg1*Δ *sfl1*Δ cells, and three independent transformants were tested for β-galactosidase activity under repressed conditions (2% glucose).

is also involved in glucose repression of *STA1* and may be a target for a repressor(s).

To isolate a multicopy inhibitor(s) that acts on UAS2, we used a method modified from that of Ahn et al. (1) (for details, see Materials and Methods). From this screening, candidate plasmids B42, C13, and C33 were isolated and sequenced. Overlapping regions of the three clones contained the same gene, *SFL1*, which is known to repress *SUC2* and *FLO11* transcription (5, 34, 37).

To examine the effect of *SFL1* on *STA1* expression in comparison to that of the previously characterized *NRG1* gene, isogenic *nrg1*Δ, *sfl1*Δ, and *nrg1*Δ *sfl1*Δ double mutants were generated. As shown in Fig. 1B, deletion of *SFL1* increased glucoamylase activity from 1.6 to 8.9 U under repressed con-

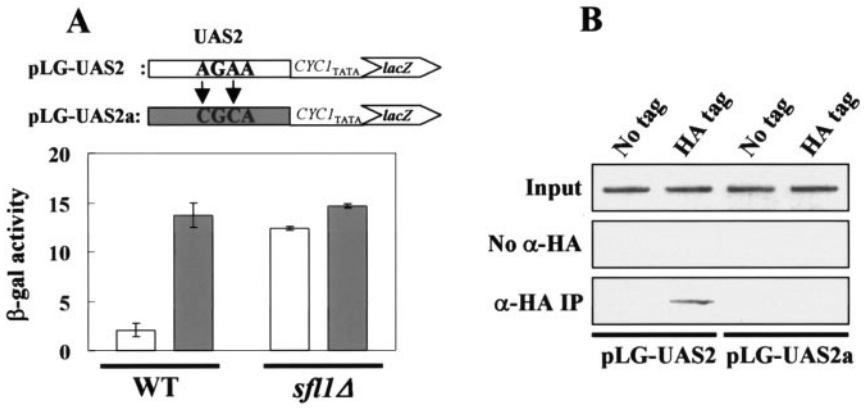

FIG. 2. Specific binding of Sfl1 to UAS2. (A). The AGAA sequences (bp −1106 to −1103) in pLG-UAS2 were replaced with CGCA by site-directed mutagenesis. The resulting construct, pLG-UAS2a, and also the parental pLG-UAS2 construct were transformed into wild type (WT) and *sfl1*Δ cells. β-Galactosidase activity was determined under repressed conditions by using three independent colonies. (B) ChIP assays for Sfl1. pLG-UAS2 or pLG-UAS2a and plasmid pRS325-*SFL1*-HA or pRS323-*SFL1*, expressing Sfl1-HA or Sfl1 from its own promoter, respectively, were cotransformed into wild-type cells. Transformants were grown to mid-log phase in synthetic medium with 2% glucose and were treated with formaldehyde to cross-link DNA and proteins. An α-HA ChIP assay was performed, and the UAS2 region was PCR amplified by using purified DNA to determine Sfll binding to UAS2.

ditions, whereas deletion of *NRG1* increased activity only twofold (from 1.6 to 3.2 U). Furthermore, *sfl1*Δ synergized with *nrg1*Δ to completely relieve glucose repression; the level of glucoamylase activity of *nrg1*Δ *sfl1*Δ cells increased under repressed conditions to 18.7 U, a level similar to that of wild-type cells under derepressed conditions (Fig. 1B). To examine whether the increased glucoamylase activity correlates with the level of *STA1* mRNA in wild-type and mutant cells, we carried out Northern blot analysis. In a glucose medium, the level of the *STA1* transcript was slightly higher in *nrg1*Δ cells than in wild-type cells, whereas it was approximately fivefold higher in *sfl1*Δ cells. Consistent with the synergistic elevation of glucoamylase activity, the *nrg1*Δ *sfl1*Δ double mutant exhibited a *STA1* mRNA level approximately 10-fold higher than that of the isogenic wild type (Fig. 1C).

Nrg1 and Sfl1 specifically bind to UAS1 and UAS2, respectively. As mentioned above, Nrg1 and Sfl1 were isolated as multicopy inhibitors acting on UAS1 and UAS2, respectively. Thus, we asked if Nrg1 and Sfl1 act on these sequences specifically. To this end, we first transformed plasmids containing UAS1-*CYC1*$_{TATA}$-*lacZ* (pLG-UAS1) or UAS2-*CYC1*$_{TATA}$-*lacZ* (pLG-UAS2) reporter genes into wild-type, *nrg1*Δ, *sfl1*Δ, and *nrg1*Δ *sfl1*Δ cells and then measured β-galactosidase activity under repressed conditions. Expression of *lacZ* from pLG-UAS1 or pLG-UAS2 in wild-type cells was very low under repressed conditions (Fig. 1D). The β-galactosidase activity of pLG-UAS1 was fourfold higher in *nrg1*Δ cells than in wild-type cells, whereas the β-galactosidase activity of pLG-UAS2 was unaffected (Fig. 1D). This suggested that Nrg1 acts on UAS1 but not on UAS2. In stark contrast, *lacZ* expression from UAS2 but not from UAS1 was derepressed about fivefold in *sfl1*Δ cells (Fig. 1D), indicating that Sfl1 specifically functions on UAS2 but not UAS1. Consistent with the synergistic increases in the level of the *STA1* transcript and the glucoamylase activity, the *nrg1*Δ *sfl1*Δ double mutant exhibited greatly enhanced β-galactosidase activities derived from UAS1 and UAS2. Taken together, these data indicate that Nrg1 specifically functions through UAS1 whereas Sfl1 is specific to UAS2.

Sfl1 acts on the heat shock elements in UAS2. It has been reported that Sfl1 has a DNA binding domain at its N terminus that is similar to that of the yeast heat shock transcription factor, and it has been proposed that Sfl1 binds to an inverted repeat, 5′ AGAA–n–TTCT 3′, of the heat shock factor element (5). Analysis of the UAS2 sequence revealed a conserved Sfl1 binding motif. To examine whether Sfl1 acts on this conserved motif, the AGAA sequence in pLG-UAS2 was replaced with CGCA by site-directed mutagenesis. The resulting plasmid, pLG-UAS2a, was transformed into wild-type and *sfl1*Δ cells, and β-galactosidase activity was determined under repressed conditions. Whereas *lacZ* expression from UAS2 was repressed in wild-type cells, *lacZ* expression from the mutated UAS2a (CGCA) was completely derepressed: the β-galactosidase activity of pLG-UAS2a (CGCA) in wild-type cells was similar to that of pLG-UAS2 in *sfl1*Δ cells (Fig. 2A). This result indicates that Sfl1 confers glucose repression by acting on the inverted repeat sequence AGAA–n–TTCT.

To examine whether Sfl1 was unable to bind to UAS2a, we performed a ChIP assay. Plasmids pRS325-*SFL1*-HA and pRS325-*SFL1*, expressing Sfl1-HA and Sfl1, respectively, were transformed into wild-type cells bearing pLG-UAS2 or pLG-UAS2a, and a ChIP assay was performed under repressed conditions. Sfl1-HA coimmunoprecipitated a UAS2 fragment, indicating that Sfl1 interacts with this fragment in vivo. Under the same condition, Sfl1-HA failed to coimmunoprecipitate UAS2a (Fig. 2B). These results indicate that Sfl1 binds to, and acts on, the conserved sequence AGAA–n–TTCT in UAS2, to repress *STA1* transcription.

Two independent UASs and URSs are involved in *STA1* expression. Both the UAS1 and UAS2 segments of the *STA1* promoter mediate glucose repression as well as activation of *STA1* expression in response to different carbon sources (Fig. 1A). These observations suggest that each of these DNA fragments acts as a UAS as well as an upstream repression sequence (URS). To determine whether UAS1 and UAS2 have independent functions as UASs and URSs, we subdivided UAS1 into UAS1-1 (−2105 to −1906) and UAS1-2 (−1905 to

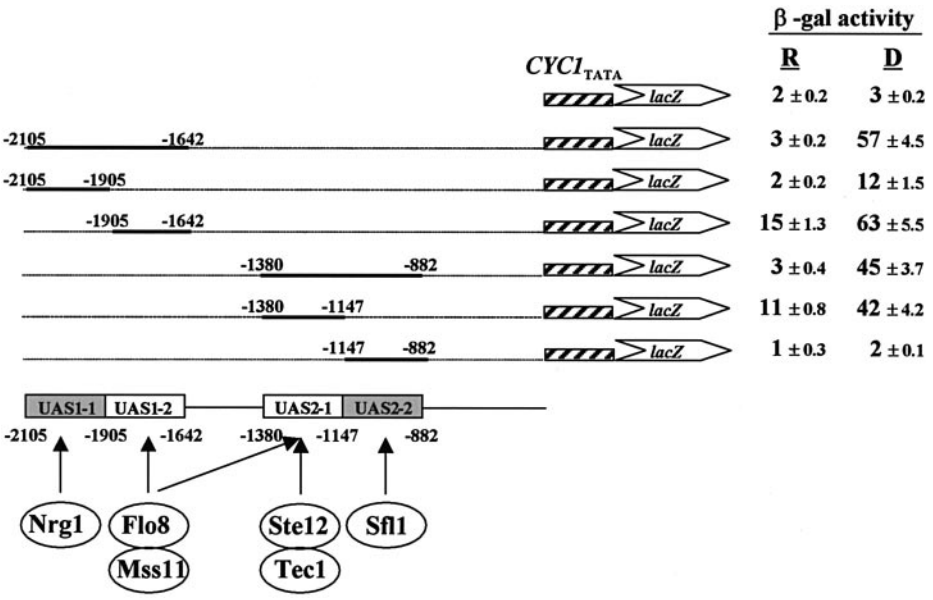

FIG. 3. Effects of carbon source on *lacZ* expression mediated by UAS1, UAS2, and their subregions. UAS1 is subdivided into UAS1-1 and UAS1-2, whereas UAS2 is subdivided into UAS2-1 and UAS2-2. Nrg1 and Sfl1 act on UAS1-1 and UAS2-2, respectively, and the transcriptional activators required for activation of UAS1-2 and UAS2-1 are indicated. The pLG-UAS series was transformed into wild-type cells, and the resulting transformants were grown to mid-log phase in synthetic medium containing 2% glucose (R) or 2% glycerol-ethanol (D). β-Galactosidase activity was determined on three independent colonies.

−1642) and UAS2 into UAS2-1 (−1380 to −1148) and UAS2-2 (−1147 to −882) (Fig. 3). As shown in Fig. 3, *lacZ* expression mediated by the complete UAS1 and UAS2 regions was totally repressed in cells grown in glucose-containing medium. However, when the Nrg1 and Sfl1 binding sequences, UAS1-1 and UAS2-2, were removed from the respective UAS1 and UAS2 regions (pLG-UAS1-2 and pLG-UAS2-1, respectively), *lacZ* was expressed even under repressed conditions. Furthermore, the β-galactosidase levels generated from UAS1-2 and UAS2-1 were similar to those generated by UAS1 and UAS2 under derepressed conditions (Fig. 3). These results demonstrate that activation of *STA1* is mediated by UAS1-2 and UAS2-1 and that the activators bind to them under both repressed and derepressed conditions. These results also suggest that UAS1-1 and UAS2-2 act as URSs mediating glucose repression of *STA1*.

Binding of Sfl1 to UAS2-2 interferes with the access of Ste12 and Tec1. Kim et al. have presented evidence that all four of the transcriptional activators Flo8, Mss11, Ste12, and Tec1 are required for activation of *STA1* expression, since deletion of any one of them abolishes *STA1* expression (21). Furthermore, it has been shown that Ste12 and Tec1 act specifically on UAS2-1, which then enhances Flo8 and Mss11 binding to UAS1-2 and UAS2-1 to activate *STA1* expression (Fig. 3) (T. S. Kim, H. Y. Kim, J. H. Yoon, and H. S. Kang, submitted for publication). These results suggest that interactions between the repressors and the upstream elements, UAS1-1 and UAS2-2, may have hindered the accessibility of the transcriptional activators to UAS1-2 and UAS2-1 and thus resulted in repression. To examine this possibility, we first constructed *FLO8*, *MSS11*, *STE12*, and *TEC1* tagged with triple HA at their C termini and integrated them into the respective genomic loci. These integrated fusion proteins were functional, since the integrated strains showed normal *STA1* expression patterns under both repressed and derepressed conditions (data not shown). To examine whether overexpressed Nrg1 or Sfl1 is able to repress *STA1* expression and inhibit binding of the activators to UASs under derepressed conditions, the integrated strains were transformed with either a multicopy *NRG1* plasmid, a multicopy *SFL1* plasmid, or an empty vector, and their glucoamylase activities were determined. As shown in Fig. 4A, *STA1* expression was repressed in cells containing the multicopy *NRG1* or multicopy *SFL1* plasmid despite the fact that they were grown under derepressed conditions. We performed a ChIP assay to test whether overexpressed Nrg1 and Sfl1 inhibit binding of the activators to the endogenous UASs of the *STA1* promoter in these strains. The multicopy *NRG1* plasmid did not affect the binding of the activators to UAS1-2 or UAS2-1 at all (Fig. 4B), indicating that overexpression of Nrg1 does not inhibit binding of the activators to the UASs even though it reduces *STA1* expression. However, the multicopy *SFL1* plasmid prevented the activators from binding to UAS2-1; UAS2-1 was hardly immunoprecipitated at all with Flo8-HA, Mss11-HA, Ste12-HA, and Tec1-HA in cells bearing the multicopy *SFL1* plasmid, whereas it was immunoprecipitated in cells containing the control vector (Fig. 4B).

We next compared the levels of the activators under the same condition to determine whether the effect of the multicopy *SFL1* plasmid was due to reduced levels of activators. Western blot analyses showed that the presence of the multicopy *SFL1* plasmid did not affect the amount of Mss11, Ste12, or Tec1 but completely abolished expression of *FLO8* (Fig. 4C). Furthermore, we revealed that the *FLO8* promoter contained the heat shock factor element recognized by Sfl1 and that Sfl1 bound to this region in vivo (data not shown). These results suggest that overexpression of Sfl1 reduces *STA1* ex-

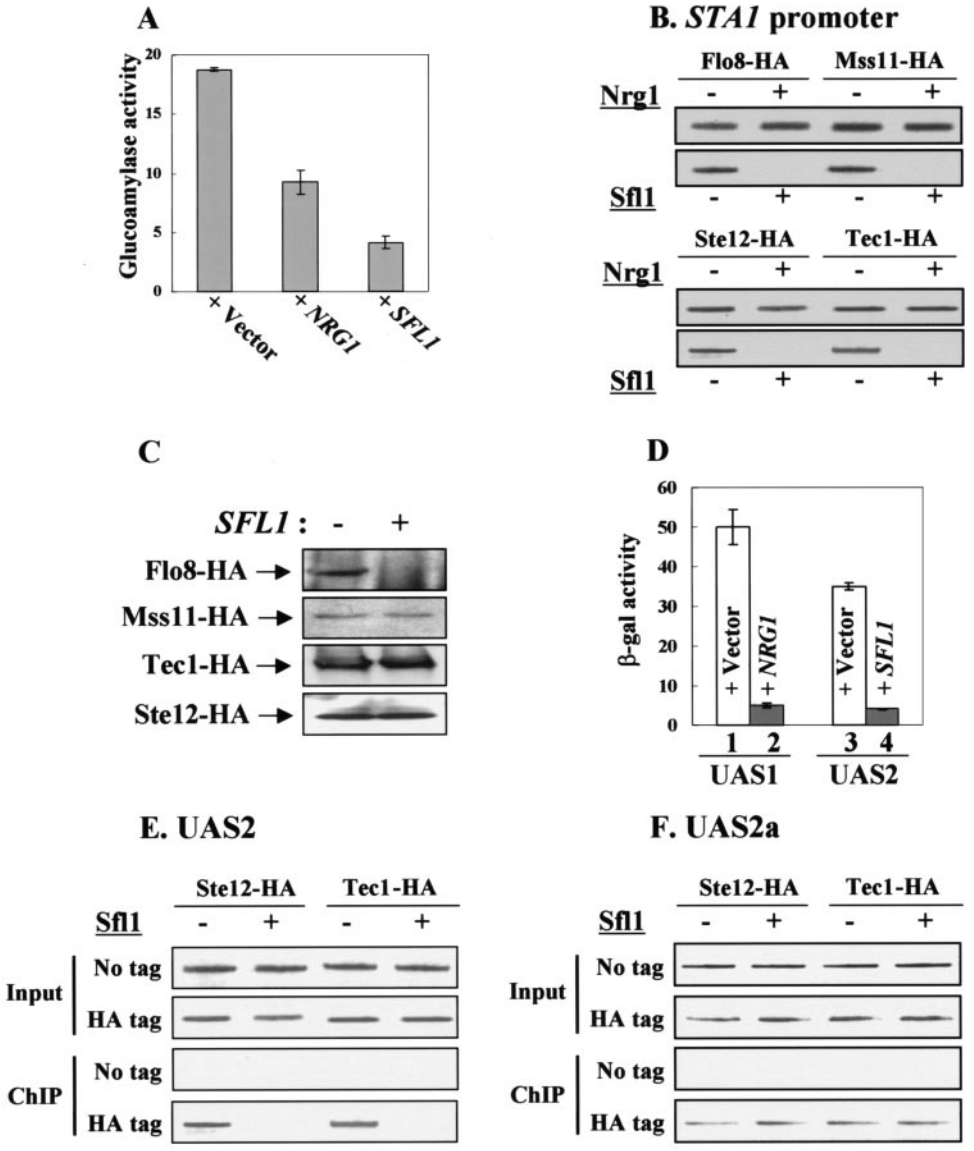

FIG. 4. Sfl1 inhibits access of Ste12 and Tec1 to UAS2-1. (A) Cells containing a control, multicopy *NRG1*, or multicopy *SFL1* plasmid were grown in 2% glycerol-ethanol medium to mid-log phase and subjected to a glucoamylase activity assay. (B) pRS323 (−), pRS323-*NRG1* (+), or pRS323-*SFL1* (+) was transformed into integrated HA-tagged strains. Transformants were grown to mid-log phase in synthetic medium containing 2% glycerol-ethanol and were fixed with formaldehyde. After α-HA ChIP, UAS1-2 and UAS2-1 were PCR amplified by using purified DNA. (C) Cells containing the multicopy *SFL1* plasmid (+) or controls (−) were grown in 2% glycerol-ethanol medium to mid-log phase, and total proteins were extracted from these cells. Levels of HA-tagged transcriptional activators from 50 or 100 μg of protein extract were determined. (D) pLG-UAS1 (lane 1), pLG-UAS1-*NRG1* (lane 2), pLG-UAS2 (lane 3), or pLG-UAS2-*SFL1* (lane 4) was introduced into wild-type cells. The resulting transformants were grown in synthetic medium containing 2% glycerol-ethanol before being subjected to a β-galactosidase activity assay. (E) pLG-UAS2 (−) or pLG-UAS2-*SFL1* (+) was transformed into wild-type, *STE12*-HA, or *TEC1*-HA cells. The resulting transformants were cultured and subjected to an α-HA ChIP assay as in panel B. (F) pLG-UAS2a (−) and pLG-UAS2a-*SFL1* (+) were transformed into the same strains. A ChIP assay was performed with an α-HA antibody as above, and Ste12 or Tec1 binding to UAS2-1 was detected by PCR.

pression via two different mechanisms: by inhibition of *FLO8* expression and also by inhibition of the binding of Ste12 and Tec1 to UAS2.

Since the binding of Ste12 and Tec1 was not affected by deletion of *FLO8* and *MSS11* (Kim et al., submitted), we next examined whether the inhibitory effect of overexpressed Sfl1 on Ste12 and Tec1 binding is direct, by using *lacZ* reporter plasmids containing either wild-type UAS2 (pLG-UAS2) or the mutant UAS2a (pLG-UAS2a). If the effect of Sfl1 is indirect, the activators will not bind to UAS2-1 in Sfl1-overexpressing cells, whether Sfl1 binds to UAS2 or not. However, if Sfl1 inhibits the binding of Ste12 and Tec1 to UAS2-1 directly, these activators will bind to UAS2a, since Sfl1 cannot bind to it. As expected, overexpression of Sfl1 repressed UAS2 on the plasmid as well as on the endogenous *STA1* promoter; the β-galactosidase activity of pLG-UAS2 was reduced by the multicopy *SFL1* plasmid but not by the control empty plasmid (Fig. 4D). Next we performed a ChIP assay. Like the endogenous

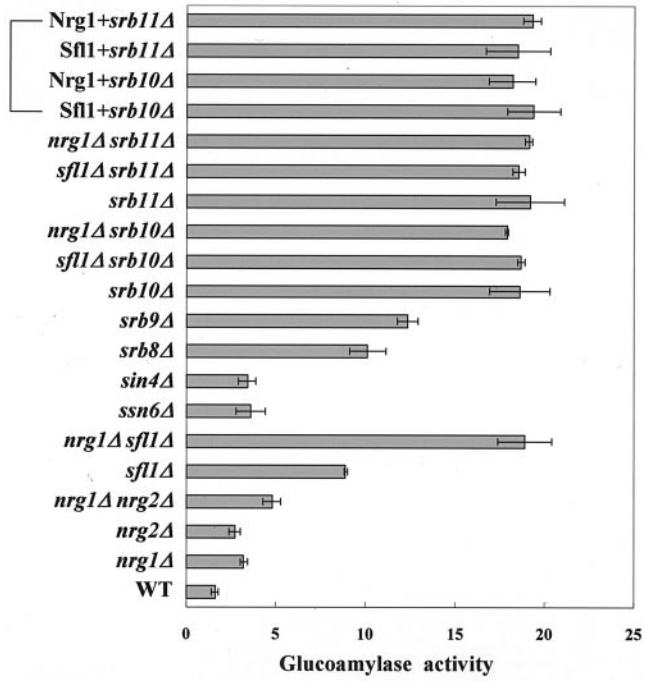

FIG. 5. Effects of Srb and mediator proteins on glucose repression of *STA1* expression. Both wild-type (WT) and mutant strains were grown in synthetic medium with 2% glucose. Nrg1+ and Sfl1+ indicate the presence of multicopy plasmids bearing *NRG1* or *SFL1* in the *srb10*Δ or *srb11*Δ cells. Average glucoamylase activities from three independent experiments are presented.

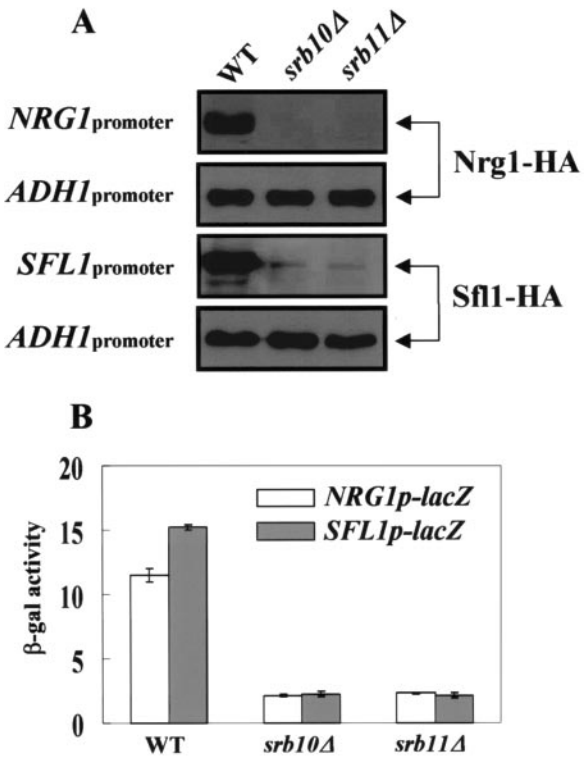

FIG. 6. The Srb8-11 complex is required for *NRG1* and *SFL1* expression. (A) Plasmids expressing Nrg1-HA or Sfl1-HA, from their own promoters or from the *ADH1* promoter, were transformed into wild-type (WT) and mutant cells. Transformants were grown to mid-log phase in synthetic medium containing 2% glucose. Protein extracts were prepared from each transformant, and 50 μg (own promoter) or 20 μg (*ADH1* promoter) of extract was separated by SDS-PAGE for immunoblotting with an α-HA antibody. The same membranes were probed with an α-actin monoclonal antibody. (B) Plasmids containing *NRG1p-lacZ* and *SFL1p-lacZ* were transformed into wild-type and mutant cells. The cells were grown in synthetic medium containing 2% glucose, and β-galactosidase activity was measured with three independent colonies.

UAS2-1, UAS2-1 on pLG-UAS2 was immunoprecipitated together with Ste12-HA and Tec1-HA in cells containing the control plasmid, but hardly at all in cells bearing the multicopy *SFL1* plasmid (Fig. 4E). However, Ste12 and Tec1 still bound to UAS2a containing the mutated Sfl1 binding site, even though *SFL1* was overexpressed (Fig. 4F). These results indicate that the binding of Sfl1 to UAS2-2 directly prevents Ste12 and Tec1 from binding to UAS2-1 to repress *STA1* expression and that the effect of Sfl1 overexpression is not an indirect consequence of altered expression of another factor(s) that may influence the binding of Ste12 and Tec1. We conclude that Sfl1 competes with Ste12 and Tec1 for occupation of UAS2.

The Srb8-11 complex is critical for glucose repression of *STA1* expression. It has been reported that the function of Sfl1 is related to that of Ssn6-Tup1 and Srb proteins, such as Srb8, Srb9, and Srb11, or to that of Sin4 (5, 37). Nrg1 also requires Ssn6-Tup1 to repress *STA1* transcription (31). We therefore disrupted the *SRB* genes, *SIN4* and *SSN6*, to investigate whether these proteins are also involved in glucose repression. In *sin4*Δ cells and *ssn6*Δ cells, *STA1* expression was slightly derepressed under repressed conditions (Fig. 5). On the other hand, glucose repression was greatly relieved in *srb8*Δ, *srb9*Δ, *srb10*Δ, and *srb11*Δ cells; notably, *srb10*Δ and *srb11*Δ completely reversed the glucose repression. Interestingly, neither *srb10*Δ nor *srb11*Δ synergized with *nrg1*Δ or *sfl1*Δ to relieve repression. In addition, the effects of *srb10*Δ and *srb11*Δ were not suppressed by multicopy plasmids bearing *NRG1* or *SFL1*, even though *NRG1* and *SFL1* were originally isolated as multicopy inhibitors of *STA1* expression under derepressed conditions (Fig. 5). Taken together, these results suggest that the function of Nrg1 and Sfl1 is closely related to that of the Srb8-11 complex.

***NRG1* and *SFL1* expression requires the Srb8-11 complex.** As mentioned above, the effect of multicopy *NRG1* and *SFL1* plasmids was not observed in either *srb10*Δ or *srb11*Δ cells, although Nrg1 and Sfl1 were isolated as multicopy inhibitors of *STA1* expression (Fig. 5). One possible explanation is that Nrg1 and Sfl1 do not function as transcriptional repressors in these deletion mutants because the Srb8-11 complex is critical for Nrg1- and Sfl1-mediated repression (5, 37). Alternatively, the failure of repression by multicopy *NRG1* and *SFL1* may have resulted from reduced levels of these repressors: the Nrg1 and Sfl1 could be unstable, or *NRG1* and *SFL1* expression might have been blocked, in *srb10*Δ or *srb11*Δ mutants.

To investigate these possibilities, we first determined the levels of Nrg1 and Sfl1 in the *srb10*Δ and *srb11*Δ mutants by immunoblotting with an α-HA antibody. Cells carrying plasmids expressing HA-tagged Nrg1 or Sfl1 from its own promoter or the *ADH1* promoter were grown in glucose medium.

Western blot analyses showed that the levels of the two repressors were substantially reduced in *srb10*Δ and *srb11*Δ cells compared to those in wild-type cells (Fig. 6A). However, when the repressors were expressed from the *ADH1* promoter, levels were almost the same in wild-type and mutant cells. These results indicate that the reduction of Nrg1 and Sfl1 levels in the *srb10*Δ or *srb11*Δ background is due not to instability but to reduced transcription. To confirm this result, we examined the activities of the *SFL1* and *NRG1* promoter regions by using a *lacZ* reporter fused to an *SFL1* or *NRG1* promoter. As expected, expression of *lacZ* from the *SFL1* and *NRG1* promoter regions was also reduced significantly in *srb10*Δ and *srb11*Δ cells from that in wild-type cells (Fig. 6B). We conclude from these results that the defect in repression by introduction of multicopy *NRG1* and *SFL1* plasmids in *srb10*Δ or *srb11*Δ cells is due to reduced Nrg1 and Sfl1 levels caused by repressed transcription of *NRG1* and *SFL1*. Thus, these results strongly suggest that the Srb8-11 complex is required to promote *NRG1* and S*FL1* expression.

Our attempt to determine whether the Srb8-11 complex directly regulates the expression of *NRG1* and *SFL1* or indirectly affects the function of the activator(s) involved in the transcription of the two repressor genes failed. However, it seems likely that the Srb8-11 complex activates expression of *NRG1* and *SFL1* indirectly, because we did not find that Srb10-HA bound to the *NRG1* or *SFL1* promoter in ChIP assays (data not shown). Nevertheless, our data show that the Srb8-11 complex functions to promote *NRG1* and S*FL1* expression.

Nrg1 and Sfl1 recruit the Srb8-11 complex to the *STA1* promoter. It has been reported that the functions of Sfl1, the Ssn6-Tup1 corepressor, and the Srb8-11 complex are closely related and that Sfl1 interacts physically with the Ssn6-Tup1 and the Srb8-11 complex (5, 22, 25, 37). We found that repression by LexA-Nrg1 also requires the Srb8-11 complex (data not shown). These results suggest that Nrg1 as well as Sfl1 interacts with the Srb8-11 complex. To examine this possibility, we performed a GST pull-down assay using yeast whole-cell lysates. Cellular lysates prepared from the integrated *SRB10*-HA strain were incubated with purified GST, GST-Nrg1, or GST-Sfl1. As shown in Fig. 7A, Srb10-HA was coprecipitated with GST-Nrg1 and GST-Sfl1 but not with GST alone.

Since our finding, together with the data reported previously (37), suggests that Nrg1 and Sfl1 may directly recruit the Srb8-11 complex to the *STA1* promoter, we performed a ChIP assay using the integrated *SRB10*-HA strain. As expected, Srb10 bound to the *STA1* promoter in wild-type cells. In contrast, Srb10 binding was marginally reduced in the *nrg1*Δ mutant and greatly diminished in the *sfl1*Δ mutant. Furthermore, the interaction between Srb10 and the *STA1* promoter was completely abolished in the *nrg1*Δ *sfl1*Δ double mutant (Fig. 7B). These results indicate that both Nrg1 and Sfl1 recruit the Srb8-11 complex to the *STA1* promoter to repress *STA1* expression.

***SFL1* expression is regulated posttranscriptionally.** It has been reported previously that *NRG1* is transcriptionally induced by glucose (31). However, it was not known whether *SFL1* transcription is also induced in the presence of glucose. Thus, we examined levels of *SFL1* mRNA in the wild-type strain under both repressed and derepressed conditions. The

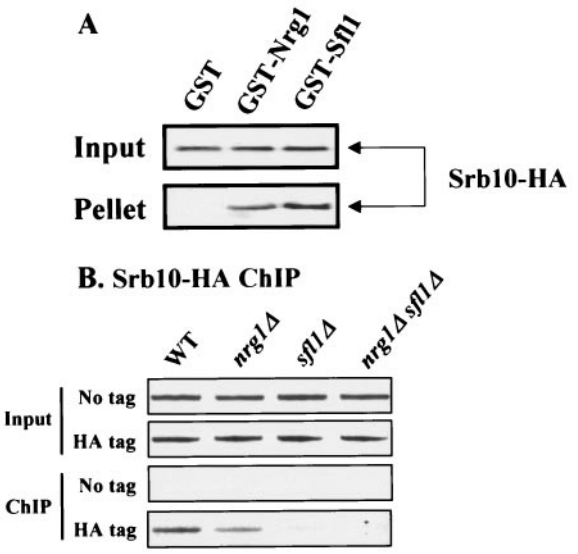

FIG. 7. Recruitment of the Srb8-11 complex to the *STA1* promoter requires Nrg1 and Sfl1. (A) Cell extracts prepared from the integrated *SRB10*-HA strain were incubated with 5 μg of GST, GST-Nrg1, or GST-Sfl1. GST proteins and their interacting proteins were precipitated with glutathione-agarose beads. Fractions of the input (1/10) and pellet (1/2) were analyzed by Western blot analysis with an α-HA antibody. (B) Cells tagged with integrated *SRB10*-HA, isogenic mutants, or nontagged strains were grown to mid-log phase in synthetic medium containing 2% glucose and were then fixed with formaldehyde. An α-HA ChIP assay was performed, and the core promoter region of the *STA1* promoter was amplified by PCR using purified DNA to detect Srb10 binding to the *STA1* promoter.

level of the *SFL1* transcript in glucose medium was similar to that in glycerol-ethanol medium (Fig. 8A). Consistent with these observations, β-galactosidase activities of the *SFL1* promoter were also similar (Fig. 8B), indicating that *SFL1* transcription is not regulated in response to different carbon sources.

Next, we examined Sfl1 protein levels in cells carrying plasmid pRS325-*SFL1*-HA, which expresses Sfl1 tagged with 3 HA molecules at its C terminus from its own promoter. This Sfl1-HA fusion was functional, since it complemented *sfl1*Δ, and the level of *SFL1*-HA transcription was not affected by glucose (data not shown). Although levels of *SFL1* transcription were similar in glucose- and glycerol-ethanol-grown cells, cells cultured in glucose medium exhibited approximately four-fold-higher Sfl1-HA protein levels than cells cultured in glycerol-ethanol medium (Fig. 8C). These data indicate that *SFL1* expression is regulated posttranscriptionally.

Since it has been reported that *NRG1* transcription is also induced during diauxic shift (7), we performed Northern blot analysis in order to investigate whether the increase in *NRG1* mRNA levels is due to the presence of glucose. Cells were grown to an OD_{600} of 1.0 in 2% glycerol-ethanol medium, and 4% glucose was then added. Figure 8D shows that transcription of *NRG1* was barely detectable in glycerol-ethanol but was initiated upon addition of glucose and continued to increase up to 24 h. About 0.5% glucose was still detected in the medium at this point, indicating that the increase in *NRG1* transcript levels is not due to depletion of the glucose. In contrast, the

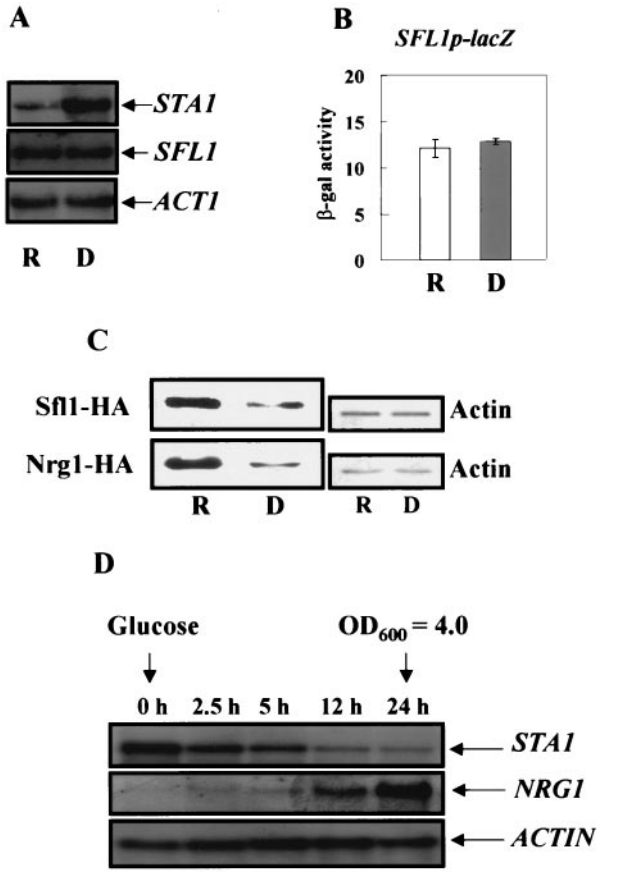

FIG. 8. Levels of Nrg1 and Sfl1 increase in the presence of glucose. (A) Wild-type cells were grown in a synthetic medium containing 2% glucose (R) or 2% glycerol-ethanol (D) as carbon sources, and total RNA was prepared for Northern blot analysis. The yeast actin gene (*ACT1*) was used as an internal control. (B) A plasmid containing *SFL1p-lacZ* was transformed into wild-type cells, and β-galactosidase activity was determined under the same conditions from three independent colonies. (C) Nrg1-HA and Sfl1-HA were expressed from their own promoters. Cells were grown in synthetic medium with 2% glucose or 2% glycerol-ethanol to mid-log phase, and total cellular proteins were prepared. Western blot analysis was performed to determine the levels of Sfl1-HA and Nrg1-HA from 50 μg of protein extract. The same membranes were probed with an α-actin monoclonal antibody. (D) Cells were grown to mid-log phase in synthetic medium with 2% glycerol-ethanol and shifted to synthetic medium with 4% glucose until they reached an OD_{600} of 4.0. Total RNA was prepared at the indicated time points for Northern blot analysis. The blot was hybridized with an *NRG1* probe and then stripped and rehybridized with *STA1* and *ACT1* probes.

STA1 mRNA level fell after 4% glucose was added. Under the same conditions, the level of Nrg1-HA is fourfold higher in glucose-grown cells than in glycerol-ethanol-grown cells. Furthermore, there was no detectable mobility shift of Nrg1-HA in either the glucose- or glycerol-ethanol-grown cells. These results suggest that glucose regulates *NRG1* at the transcriptional level, whereas it regulates *SFL1* at the posttranscriptional level.

DISCUSSION

In this study, we provide evidence that the transcriptional repressors Nrg1 and Sfl1 and the members of the Srb8-11 complex are required for glucose repression of *STA1* expression. Our data showed that Sfl1 acts specifically on UAS2 of the *STA1* promoter, indicating that this region contains a binding site for Sfl1. It has been reported that Sfl1 has an N-terminal DNA binding domain similar to that of the yeast heat shock transcription factor (5, 10). Based on these observations, Conlan and Tzamarias proposed that Sfl1 might bind to an inverted repeat of the heat shock factor element, 5′ AGAA–n–TTCT 3′, and later showed that Sfl1 bound to the region from −475 to −316 of the *FLO11* promoter, containing a putative heat shock factor element, in vivo (5). However, they did not confirm whether Sfl1 directly interacts with this conserved binding element or not. Recently, Pan and Heitman reported that Sfl1 bound directly to the region from −1400 to −1150 of the *FLO11* promoter in vitro (30). However, even though this region is very similar to the region from −1316 to −1088 of the *STA1* promoter, except for a 20-bp insert, 2-bp deletions, and a few small substitutions, we failed to find the heat shock element in this region. Instead, we found that a heat-shock factor element ($5' \ ^{-1106}$AGAA–*n*–TTCT$^{-1034,-975,-954,-919}$ 3′ [n = 64, 123, 144, and 179 bp]) exists in UAS2-2 (−1147 to −882) and appears to be critical for in vivo Sfl1 binding and for glucose repression of *STA1*.

Previous reports showed that the activators, such as Flo8, Mss11, Ste12, and Tec1, are required for activation of *STA1* and *FLO11* expression (11, 12, 13, 21). Flo8 and Sfl1, which antagonistically control *FLO11* expression, are direct targets of PKA (30). Phosphorylation by the PKA catalytic subunit Tpk2 activates *FLO11* transcription by inhibiting Sfl1 binding and promoting Flo8 binding to the region from −1400 to −1150 of the *FLO11* promoter. It has been suggested that Sfl1 competes with the activator Flo8 for occupation of this region (30). The 5′ upstream regions of the *FLO11* and *STA1* genes are quite similar, and these genes are coregulated in response to environmental signals (12). Thus, we speculated that *STA1* expression might be regulated positively or negatively by Tpk2 and that Sfl1 and Flo8 might compete with each other to occupy the *STA1* promoter. However, *STA1* expression was not affected by deletion of *TPK2* (unpublished data). Furthermore, we failed to observe that Sfl1 competes with Flo8 for binding to the *STA1* promoter. Rather, the Sfl1 repressor appears to inhibit *FLO8* expression. We have evidence that the level of Flo8 is reduced in glucose-grown cells (Kim et al., submitted). Here we also show that Sfl1 overexpression reduces *FLO8* expression (Fig. 4). In fact, we reveal that the *FLO8* promoter contains the heat shock factor element and that Sfl1 binds to the *FLO8* promoter in vivo. These results indicate that Sfl1 represses *FLO8* expression, and *FLO8* is a new target of Sfl1. On the other hand, we also present direct evidence that the repressor Sfl1 competes with the activators Ste12 and Tec1 to occupy UAS2 of the *STA1* promoter. Thus, our results indicate that Sfl1 represses *STA1* expression via two distinct mechanisms: by directly preventing the binding of Ste12 and Tec1 to UAS2-1 and by repressing the expression of *FLO8*.

In UAS1, in contrast, competition between the repressor and activators was not observed (data not shown). Thus, Nrg1 binding to UAS1-1 does not prevent Flo8 and Mss11 from binding to the UAS of the *STA1* promoter, and a different mechanism must exist to account for the Nrg1-dependent repression. We suggest that Nrg1 recruits a general corepressor,

such as Ssn6-Tup1, perhaps together with Srb proteins, and that this inhibits the interaction between the transcriptional activators and their coactivators or RNA polymerase II. It is also possible that Tup1, which is recruited by Nrg1, alters the chromatin structure of the *STA1* promoter.

Mutants with mutations of *SRB* genes, such as *SRB8*, *SRB9*, *SRB10*, and *SRB11*, have significantly increased glucoamylase activity and hyperinvasive phenotypes (Fig. 5) (data not shown). In contrast, mutation of *SIN4*, a component of the RNA polymerase II subcomplex that interacts physically with Sfl1, also induces the hyperinvasive phenotype (data not shown), but the glucoamylase activity of this mutant is only marginally elevated under repressed conditions (Fig. 5). These results indicate that although *STA1* and *FLO11* are to a large extent coregulated, certain factors act differentially on them. The functions of Nrg1 and Sfl1 are closely related to those of the Ssn6-Tup1 and the Srb8-11 complex (5, 31, 37). Furthermore, repression by LexA-Ssn6 and LexA-Tup1 requires the Srb8-11 complex (22, 25). However, our findings suggest that the Srb8-11 complex is more important than Ssn6-Tup1 in glucose repression of *STA1* expression.

We also showed that the Srb8-11 complex was required for expression of both *NRG1* and *SFL1*, though the molecular mechanism by which it mediates this regulation is not clear at present. Since we did not find that Srb10-HA bound to the *NRG1* or *SFL1* promoter (data not shown), it is possible that the Srb8-11 complex positively regulates transcriptional activators that are involved in *NRG1* and *SFL1* transcription. Previous reports showed that Srb10 both positively and negatively regulates gene-specific activators, such as Gal4, Sip4, Gcn4, and Ste12 (3, 18, 28, 39). The transcriptional activation activity of Sip4 is stimulated by Srb10, but Srb10 phosphorylates Ste12 and Gcn4 and inhibits their function by promoting their turnover. Thus, in glucose-grown cells, the Srb8-11 complex represses the transcription of *STA1* by activating *NRG1* and *SFL1* expression. Furthermore, we found that the interaction between Srb10 and the *STA1* promoter required functional Nrg1 and Sfl1 (Fig. 7), strongly suggesting that the Srb8-11 complex is recruited to the *STA1* promoter by Nrg1 and Sfl1. Our data suggest that the Srb8-11 complex plays essential roles in glucose repression of *STA1* expression by activating *NRG1* and *SFL1* expression and also by participating in Nrg1- and Sfl1-dependent repression (Fig. 9).

We showed that the level of Sfl1 was higher in glucose-grown cells than in glycerol-ethanol-grown cells, although *SFL1* transcription is neither significantly induced nor repressed with different carbon sources. In addition, an increased dosage of Sfl1 by a multicopy plasmid reduces *STA1* expression, even under derepressed conditions. Thus, it is likely that the level of Sfl1 is a more important factor in glucose repression of *STA1* than its modification. However, the question of whether another protein kinase besides Tpk2 also regulates Sfl1 function or not remains to be investigated.

Snf1 kinase was known to interact with the Nrg1 repressor and to act as an antagonist of Nrg1 (23, 41). Interestingly, it has been shown that Nrg1 localization is not affected by different carbon sources (41). However, the molecular mechanism by which Snf1 regulates the function of Nrg1 is largely unknown. It was observed previously that induction of *NRG1* transcription was enhanced about 6-fold by glucose (31), but it was also

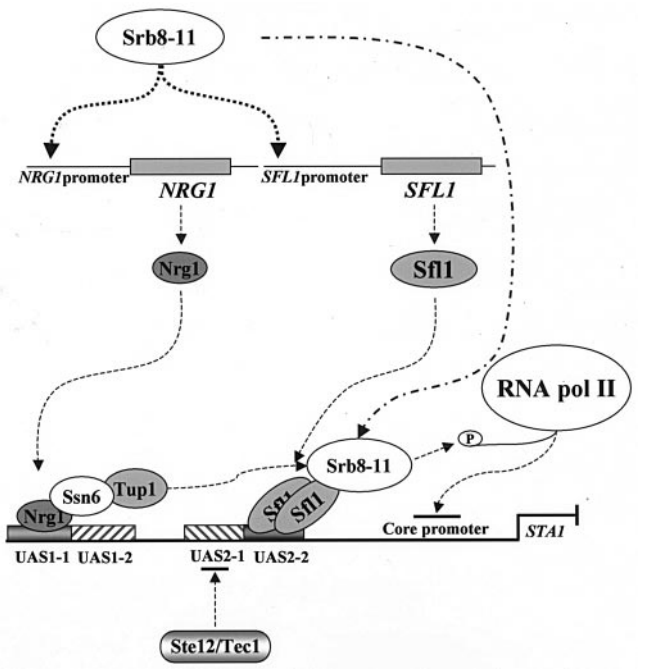

FIG. 9. Model of the molecular mechanism of glucose repression of *STA1* expression. In glucose-grown cells, the Srb8-11 complex activates *NRG1* and *SFL1* expression. The increased levels of Nrg1 and Sfl1 bind to UAS1-1 and UAS2-2, respectively. DNA-bound Nrg1 and Sfl1 recruit the Ssn6-Tup1 corepressor and the Srb8-11 complex to the *STA1* promoter. In addition, DNA-bound Sfl1 prevents access of transcriptional activators Ste12 and Tec1 to UAS2-1. The Ssn6-Tup1 or Srb8-11 complex may alter chromatin structure or phosphorylate the C-terminal domain of RNA polymerase II prior to the formation of an initiation complex.

reported to be enhanced 2.7-fold during a diauxic shift (7). In the present work, we showed that *NRG1* transcription was induced after glucose addition but before there was any glucose depletion (Fig. 8D). Furthermore, the level of Nrg1 is also about fourfold higher in glucose-grown cells than in glycerol-ethanol-grown cells (Fig. 8C). These data suggest that *NRG1* expression is induced by glucose, although it is also induced during a diauxic shift. In addition, the levels of Flo8 and Tec1 that are required for activation of *STA1* expression are significantly reduced in the presence of glucose (Kim et al., submitted). We therefore suggest that the increased quantities of Nrg1 and Sfl1 and the reduced levels of Flo8 and Tec1 repress the transcription of *STA1* and *FLO11* in glucose-grown cells.

In conclusion, glucose repression of *STA1* expression requires a series of complex regulatory elements (Fig. 9). The Srb8-11 complex activates *NRG1* and *SFL1* expression under repressed conditions; Nrg1 binds directly to UAS1-1, whereas Sfl1 binds to UAS2-2 after forming multimers through the coiled-coil domains. When bound to their specific sites, Nrg1 and Sfl1 can recruit the Ssn6-Tup1 corepressor or the Srb8-11 complex, resulting in alteration of the chromatin structures and/or perhaps hindering the formation of the initiation complex on the *STA1* promoter. Furthermore, Sfl1 represses *FLO8* expression and inhibits the access of Ste12 and Tec1 to the *STA1* promoter.

ACKNOWLEDGMENTS

We thank Kyung S. Lee (NIH) for preparation of the manuscript.

This work was supported by a grant from the Korea Science and Engineering Foundation [R01-2002-000-00221-0(2002)]. T. S. Kim and S. B. Lee are supported by a BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

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