NGG1p/ADA3p is a yeast dual function regulator required for the complete glucose repression of GAL4p-activated genes (Brandl, C. J., Furlanetto, A. M., Martens, J. A., and Hamilton, K. S. (1993) EMBO J. 12, 5255-5265). Evidence for a direct role for NGG1p in regulating activator function is supported by the finding that NGG1p is also required for transcriptional activation by GAL4p-VP16 and LexA-GCN4p (Pina, B., Berger, S. L., Marcus, G. A., Silverman, N., Agapite, J., and Guarente, L. (1993) Mol. Cell. Biol. 13, 5981-5989). By analyzing deletion derivatives of the 702-amino acid protein, we identified a region essential for glucose repression within residues 274-373. Essential sequences were further localized to a segment rich in Phe residues that is predicted to be an amphipathic α helix. As well as finding mutations within this region that reduced glucose repression, we identified mutations that made NGG1p a better repressor. In addition, NGG1p probably represses GAL4p activity as part of a complex containing ADA2p because single and double disruptions of ngg1 and ada2 had comparable effects on glucose repression. We also localized a transcriptional activation domain within the amino-terminal amino acids of NGG1p that is proximal or overlapping the region required for glucose repression. Activation by GAL4p-NGG1p1–373 requires ADA2p; however, activation by GAL4p-NGG1p1–308 is ADA2p-independent. This suggests that a site required for ADA2p interaction lies between amino acids 308 and 373 and that ADA2p has a regulatory role in activation by GAL4p-NGG1p1–373.

The genes required for galactose metabolism in Saccharomyces cerevisiae provide one of the principal model systems for the interfacing between positive and negative transcriptional regulatory networks. In response to galactose, these genes are induced approximately 1000-fold in a process that requires the GAL4p transcriptional activator protein (reviewed in Ref. 1). This induction requires signaling through GAL3p, which in turn results in the dissociation or conformational change of an otherwise inactive GAL4p-GAL80p complex (2-10).

Transcriptional activation of the GAL genes is completely blocked by glucose in a rapid process (reviewed in Refs. 11-14). A number of direct mechanisms for glucose repression have been identified, including the regulation of GAL4 expression in glucose medium. Binding of MIG1p (15) to the GAL4 promoter results in decreased transcription of GAL4 of approximately 5-fold in glucose-containing medium (16, 17). This 5-fold decrease in GAL4 expression is amplified to give a decrease in GAL gene transcription of approximately 100-fold (16, 18). In addition, a number of glucose-responsive negative regulatory elements (URS elements) are found within the GAL1-10 promoter (19-24) that account for an approximately 3-fold decrease in expression (18).

The effect of reduced GAL4p expression will not be evident until the turnover of previously expressed GAL4p, a process that occurs in the order of hours (25). Mechanisms in addition to URS-mediated repression must exist to generate the 6-10-fold repression of GAL1 expression seen 10 min after the addition of glucose (18). A GAL80p-dependent mechanism for GAL4p inactivation was described by Lamphier and Ptashne (21). In addition, Stone and Sadowski (26) found that the central region of GAL4p is required for maximal glucose repression, suggesting a role for this domain as a target for inactivation. Furthermore, we isolated NGG1p (also called ADA3; Ref. 27) based upon its involvement in the glucose repression of GAL10 (28). ngg1 was identified as a recessive null mutation that in the presence of a gal80 background resulted in a 300-fold relief of glucose repression for the GAL10-related promoter his3-G25. Approximately 10-fold of this relief of glucose repression was attributable to ngg1.

GAL4p is the most likely target for NGG1p action based upon several observations (28). Relief of glucose repression by ngg1 was dependent on GAL4 but was independent of the GAL4 promoter. NGG1p thus does not appear to act by regulating transcription of GAL4. Repression by NGG1p was observed for promoters containing independent GAL4p binding sites, thus excluding a URS-dependent mechanism. Direct action of NGG1p on the function of transcriptional activators was also suggested by the finding that nonfunctional mutations of ngg1 suppress the lethal effects of overexpression of GAL4p-VP16 fusions (29). This suppression was due to reduced activation by GAL4p-VP16 in this background. Subsequently, Pina et al. (27) and Georgakopoulos et al. (30) demonstrated that NGG1p is required for maximal transcriptional activation by a group of activators that includes GAL4p-VP16 and LexA-GCN4.

Genetic and biochemical evidence suggests that NGG1p acts in a complex with at least two additional proteins, ADA2p and GCN5p (27, 30-32). ada2 was also identified by its ability to suppress the toxic effects of overexpression of GAL4p-VP16 (29). Mutations in gcn5 were isolated by their ability to reduce transcriptional activation by GCN4p (33). Individually ADA2p, GCN5p, and NGG1p can activate transcription when tethered to the promoter by a DNA binding domain (30-32). A functional relationship between these proteins is also consistent with the finding that in all the combinations analyzed, this
transcriptional activation requires the presence of the other proteins. In addition, ada2, ngg1, and gcn5 mutant strains all show similar slow growth phenotypes and reduced transcriptional activation, with double mutants having no more severe a phenotype (27, 28, 30–32). Association of the carboxy-terminal 250 amino acids of NGG1p with ADA2p was shown by far Western blotting and immunoprecipitation (32). The association of NGG1p and GCN5p is indirect, requiring ADA2p as a bridge (31, 32). Based on the above evidence, Horiiuchi et al. (32) have proposed that an ADA complex including NGG1p, ADA2p, and GCN5p serves to functionally link the transcriptional activator protein with the basal transcriptional machinery. This model is supported by the finding that ADA2p interacts directly with VP16 (34, 35), GCN4p (35), and GAL4p (36). Whereas the downstream target for the complex is unknown, the findings that the transcriptional defect of ada2 strains can be observed in vitro (29) and that ADA2p from crude yeast extracts is retained on TBP affinity columns (35) suggest that the target may be TBP, although an interaction with a second basal factor or with a nucleosomal component cannot be excluded.

To initiate studies into the mechanism of glucose repression by NGG1p, we have begun to analyze the structure/function of NGG1p by using a complex that includes ADA2p, and GCN5p serves to functionally link the transcriptional activator protein with the basal transcriptional machinery. This model is supported by the finding that ADA2p interacts directly with VP16 (34, 35), GCN4p (35), and GAL4p (36). Whereas the downstream target for the complex is unknown, the findings that the transcriptional defect of ada2 strains can be observed in vitro (29) and that ADA2p from crude yeast extracts is retained on TBP affinity columns (35) suggest that the target may be TBP, although an interaction with a second basal factor or with a nucleosomal component cannot be excluded.

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Materials and Methods

Yeast Strains, Media, and Growth Conditions—Yeast strain CY756 (Ref. 28; relevant genotype, MATa ura3-52 ade2-101 trp-11 lys2-801 his3-d200 leu2-32-PET56 ngg1::TRP1) is a derivative of KY270 (37), which contains a Tn10 LUK (38) disruption of the his3 gene. CY756 was kindly supplied by Dr. S. Elledge. CY922 is a derivative of this strain made ura- by using selection on 5-fluoroorotic acid. wt, wild type.

| Strain | Background | ngg1 | URA3 | gal108 | ad4 | ade2 |
|--------|------------|------|------|--------|-----|------|
| CY756  | CY320      | ngg1::TRP1 | wt | wt | wt |
| CY914  | CY320      | ngg1::TRP1 | null | wt | wt |
| CY922  | Y190       | wt    | null | null | wt |
| CY99   | CY320      | wt    | null | wt | wt |
| CY927  | CY320      | wt    | null | wt | Tn10 LUK |
| CY939  | CY320      | ngg1::TRP1 | null | wt | Tn10 LUK |
| pDMY  | CY320      | DMYC-ngg1 | null | wt | Tn10 LUK |

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shown are from a minimum of three independent experiments with a standard error of not more than 30%.

DNAs representing the GAL4-NGG1 fusions in pAS1 were introduced into yeast strain CY922 containing his3-G4 lacZ, a derivative of the his3 promoter that contains five optimal GAL4 binding sites in the position normally occupied by the GCN4p binding site (Ref. 28; see Fig. 1A) or his3-G25 lacZ, a derivative of the his3-G4 lacZ, a derivative of the his3 promoter that contains five optimal GAL4 binding sites in the position normally occupied by the GCN4p binding site (Ref. 28; see Fig. 1A) or his3-G25 lacZ. Activation by the GAL4p-NGG1p fusions was determined by measuring β-galactosidase activity using chlorophenol red-β-D-galactopyranoside as substrate as determined by Durfee et al. (51) after disruption of cells with glass beads (54). Alternatively, activation by the GAL4p-NGG1p fusions was determined by the relative growth rate of the strains on minimal plates containing 50 mM 3-amino-1,2,4-triazole (AT) because yeast strain CY922 contains an integrated GAL-HIS3 reporter fusion (see Fig. 1). As his3 is expressed from the GAL1 promoter and growth in the presence of AT is directly related to the level of his3 mRNA (37, 55), growth rate in AT is related to activity of the GAL4p-NGG1p fusions. The negative control GAL4p-p53 was provided by Dr. S. Elledge.

Western Blot Analysis—Total protein was isolated from 10-ml cultures of yeast strains grown in minimal medium containing 2% glucose by glass bead disruption as described by Gill et al. (56). Protein concentration was calculated in these crude lysates using the procedure of Bradford (57). 200 μg of total protein was separated by SDS-polyacrylamide gel electrophoresis on 7.5% or 10% polyacrylamide gels (58). Gels were transferred to nitrocellulose by semi-dry blotting with carbonate buffer (59) for 2.5 h at 1 mAmper/cm. Myc-tagged proteins were detected using the SuperSignal kit (Pierce) and a primary antibody from Ascites fluid derived from the Myc1–9E10 cell line (46). The primary antibody was used at a dilution of 1:2000 after being preabsorbed to control yeast protein at a ratio of 1.0 μl of antibody/30 μg of protein for 1 h on ice. Blocking with 5% casein and antibody incubations were performed in Tris-buffered saline containing 0.1% Tween-20. The filter was washed after each antibody by incubation with Tris-buffered saline containing 0.1% SDS, 0.5% Triton X-100, and 0.5% mEdTA.

Protein Structure Prediction and Computer Searches—Protein structure predictions were performed using the PHD program on the PredictProtein server at EMBL (60–62). Similarities of NGG1p with sequences in the SwissProt data base were determined using the Blast program at the NCBI (63).

RESULTS

Regions of NGG1p Required for Glucose Repression—To initially identify the regions of NGG1p required for glucose repression, we analyzed deletion derivatives of the gene for their ability to repress expression of GAL10 in glucose medium. NGG1 alleles were introduced into yeast in the vector pDMYC, which allows the integration of Myc-tagged NGG1p at the his3 locus. We placed NGG1 under the control of the constitutive ded1 promoter (64) because the introduction of functionally different ngg1 alleles may directly or indirectly affect its own expression (Fig. 1). We chose to integrate the NGG1 alleles into the genome at his3, which allows for virtually complete complementation of the null allele in comparison with the incomplete complementation, which was found when NGG1 was introduced into yeast on a centromeric plasmid (not shown).

Deletion derivatives were integrated into yeast strain CY914, which contains disruptions of ngg1 and gal80, and shows an approximately 10-fold decrease in glucose repression of the GAL10 related his3-G25 promoter (28). The ability of the NGG1p deletion derivatives to repress expression of the his3-G25 lacZ reporter fusion (Fig. 1B) and their relative expression as detected by Western blotting are shown in Fig. 2. In this experiment disruption of NGG1 resulted in a 7.7-fold decrease in glucose repression in comparison with a wild type NGG1 allele expressed from the ded1 promoter (compare ngg1 wild type with ngg1).

The analysis of amino- and carboxyl-terminal deletions of NGG1p was hampered by the apparent instability of these molecules. Deletion of the carboxyl-terminal 21 or 31 amino acids (ngg1(1–641) and ngg1(1–671)) had a minimal effect on transcriptional repression by NGG1p. Further deletion of the carboxyl terminus to amino acid 645 or beyond resulted in a total loss of function (not shown); however, a direct functional role for these carboxyl-terminal sequences cannot be concluded because NGG1p was not detectable by Western blotting in extracts from cells expressing these derivatives. Deletion of the amino-terminal 52 amino acids resulted in a 3-fold decrease in activity (ngg1(3–302)). Similar to deletions at the carboxyl terminus, this decrease may be totally or in part explained by a decrease of greater than 4-fold in expression of the protein (Fig. 2B). This decrease in NGG1p expression was seen for all amino-terminal deletions. Further deletion to aminoacid 241 (ngg1(1-242)) had minimal effect on function. However, a functional role for sequences carboxyl-terminal to amino acid 242 was suggested because deletion to amino acid 301 (ngg1(1–302)) and 307 (ngg1(1–307)) resulted in a virtually complete loss of glucose repression by NGG1p.

To further delineate the region around amino acid 300 essential for function, two internal in frame deletions were constructed, ngg1(1237–303) and ngg1(303–372). Both deletions resulted in almost total loss of repression by NGG1p while having approximately equivalent levels of expression as compared with the wild type. These internal deletions thus define at least one region including residues from amino acids 274 to 373 that is required for glucose repression.

The region surrounding amino acid 300 is rich in Phe residues, containing five Phe residues over a 12-amino acid stretch (27, 28). Similar Phe-rich regions are found in a group of diverse proteins including the yeast proteins KEX1p and HAP1p and HIV-gag (27). We have analyzed the region of amino acids 236–375 using the PHD program from the PredictProtein server (60–62) to search for additional alignments that may provide a clue to function and to predict secondary structure (Fig. 3A). Although no close structural homologies were detected for this region, the sequence from Gin to
null strain CY914. Sequence analysis of the random mutants, resulted in 4-fold reductions in expression having a range of \( \beta \)-galactosidase activity. Eleven strains analyzed had no NGG1p activity and were comparable with the null strain CY914. Sequence analysis of the ngg1 alleles expressed in these strains revealed that each contained a deletion or insertion resulting in a frameshift. Because of the high frequency of such mutations in the pool of nonfunctional mutants, no additional alleles of this class were analyzed. The sequences of the remaining alleles are shown in Fig. 4. As mentioned above, ngg1 is unable to fully complement when introduced in a centromeric plasmid. When introduced into CY914 on centromeric plasmids, the wild type allele or ngg1R5, an allele with the Leu\(^{274} \rightarrow \text{Gln} \) change found in most of the randomized alleles, resulted in 4-fold reductions in expression of his3-G25 lacZ. This compares with the approximate 10-fold effect when NGG1 is integrated. The reason for this difference in activity when NGG1 is not known, but Western blot analysis suggests that it is not related to differences in protein levels (not shown).

Three observations can be made from the analysis of the random mutants. First, similar to NGG1D274-307, multiple amino acid changes in this region (for example R58, R56, R10, R26, and R85) can result in weakly functional NGG1p derivatives. Of these, the expression and activity of NGG1pR56 and double stranded by mutually primed synthesis (49). Mutations were incorporated at a 6% frequency in the coding regions for Leu\(^{274} \rightarrow \text{Pro}^{274} \) and Ile\(^{283} \rightarrow \text{Phe}^{283} \). Lys\(^{299} \rightarrow \text{Gln}^{299} \) were not mutated at a high frequency because they provide the annealing site in the mutually primed synthesis reaction. The ngg1 alleles were introduced on the URA3 centromeric plasmid YCp88 into yeast strain CY914 containing the his3-G25 lacZ reporter fusion. Ura\(^+ \) transformants were screened for \( \beta \)-galactosidase activity on minimal plates containing X-gal, as a measure of NGG1p function. Thirty ngg1 alleles were isolated from strains having a range of \( \beta \)-galactosidase activity. Eleven of the strains analyzed had no NGG1p activity and were comparable with the null strain CY914. The reason for this difference in activity when NGG1 is not known, but Western blot analysis suggests that it is not related to differences in protein levels (not shown).

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The importance of residues within the first putative helix was not only found to disrupt/enhance function with a random selection protocol led to the identification of a Putative helix only Phe298 mutations analyzed at four different amino acids in the first helix. Having shown that residues within the first helix are important, we next tested the activity of directed mutations (Fig. 5). Surprisingly, conversion of Ala300 to the strong helix breaker residues Lys resulted in a significant increase in repression (not shown). Second, amino acid changes (Fig. 5A). These alleles were isolated from two independent clones. Standard errors for other derivatives were not more than 30%.

NGG1pAS3 were analyzed after cloning into pDMYC and integration into the genome. Under these conditions, both were expressed at a level comparable with that of the wild type as determined by Western blotting but have approximately one-sixth the activity in glucose repression (not shown). Second, many of the functional alleles (R6, R16, and R3, for example) contained multiple point mutations. This as well as the finding that no weakly functional alleles were identified that encoded proteins with single or double amino acid changes suggests that the region is quite tolerant to amino acid changes. Third, four alleles (R12, R21, R22, and R63) were identified that had significantly more activity in glucose repression than the wild type. Consistent with the absence of the protein as shown by Western blot analysis, these amino acids could not be clearly confirmed with this method. The solid line divides two independent experiments. Ratio to wt is the ratio of β-galactosidase activity for the NGG1p derivative as compared with the wild type protein. B, Western blot analysis of NGG1p derivatives. Isolation of protein and Western analysis from strains expressing the indicated Myc-tagged NGG1p derivatives was as described under "Materials and Methods" and in the legend to Fig. 2. 50 μg of protein was analyzed for all samples except for 10 μg for the wild type sample shown in lane 5. CY99 is a control strain that contains untagged NGG1p.

These alleles included Phe298, the only residue not found with at least one mutation in the random selection experiment. Consistent with the lack of functional change seen with single amino acid changes in the random selection experiment of the seven point mutations analyzed at amino acids 294–302, substitution at amino acids 304–307, ngg1AS3, resulted in approximately 5- and 3-fold losses of NGG1p activity, respectively. Loss of repression was not due to the absence of the protein as shown by Western blot analysis (Fig. 5B). ngg1AS3 was constructed to examine the role of residues within the second putative helix. A functional role for these amino acids could not be clearly confirmed with this mutation because it resulted in less than a 50% loss of activity. Having shown that residues within the first helix are involved in glucose repression, we analyzed molecules with single amino acid changes (Fig. 5A). Included in this group were mutations of Phe298, the only residue not found with at least one mutation in the random selection experiment. Consistent with the lack of functional change seen with single amino acid changes in the random selection experiment of the seven point mutations analyzed at different amino acids in the first putative helix only Phe298 → Lys resulted in a significant reduction (approximately 2-fold) in glucose repression by NGG1p. The repression domain found within this region, like the activation domains of many activator proteins, thus seems to largely depend upon its overall structure rather than on any single amino acid. Surprisingly, conversion of Ala300 to the strong helix breaker

| Allele | Amino acid sequence | lacZ Units |
|--------|---------------------|------------|
| wt     | DLLPGLPDMFSHPKPTDG | 27±5       |
| R2     | DQLPGLPDMFSHPKPTDG  | 25         |
| R5E    | KHTSRLP             | 80         |
| R5E    | HHYSP              | 59         |
| R10    | LKA                | 64         |
| R9E    | SRH                | 50         |
| R8E    | EDC                 | 36         |
| R1     | RL                  | 49         |
| R1     | LTV                | 49         |
| R7E    | EDC                 | 46         |
| R7A    | NSV                | 36         |
| R6     | SE                 | 34         |
| R6     | S                  | 34         |
| R6     | FLLN               | 29         |
| R3     | T                  | 27         |
| R12    | R                   | 20±4       |
| R21    | K                  | 19±1       |
| R22    | I                  | 19±2       |
| R63    | R                   | 17±3       |

**FIG. 4.** Glucose repression of his3-G25 by random mutations within amino acids 274–307 of NGG1p. Random mutations were generated within the coding sequence for amino acids 274–307 by mutually primed synthesis (50). These alleles, expressed from the his1 promoter, were introduced into yeast strain CY914 (ngg1 Δhis3-108) on the URA3 centromeric plasmid YCp88 (43). Transformants were plated on minimal medium containing 2% glucose and X-gal, with colonies displaying a range of expression of a his3-G25 lacZ reporter selected for further analysis. Plasmids were recovered from these strains, and the mutagenized region was sequenced. The plasmids were then reintroduced into CY914 containing his3-G25 lacZ. β-Galactosidase activity was determined for these strains using O-nitrophenyl-β-D-galactopyranoside as a substrate after growth in minimal medium containing 2% glucose. Amino acid changes are indicated in Fig. 5A with mutations desired for cells grown in minimal medium containing 2% glucose. Amino acid changes are indicated in bold and underlined. The solid line divides two independent experiments. Ratio to wt is the ratio of β-galactosidase activity for the NGG1p derivative as compared with the wild type protein. B, Western blot analysis of NGG1p derivatives. Isolation of protein and Western analysis from strains expressing the indicated Myc-tagged NGG1p derivatives was as described under "Materials and Methods" and in the legend to Fig. 2. 50 μg of protein was analyzed for all samples except for 10 μg for the wild type sample shown in lane 5. CY99 is a control strain that contains untagged NGG1p.
proline did not affect NGG1p function. It should be noted, however, that this substitution results in only a minor change in the helix potential of the region as predicted from the PHD program, despite the occurrence of this residue at the central position of the putative helix.

Two mutations, Phe295 → Ala and Gin294 → Lys, resulted in increased activity of NGG1p in glucose repression. This effect was similar to that seen for ngg1p294, ngg1p221, and ngg1p222, and Gin294 → Lys was in fact constructed to determine if this amino acid change was the contributing factor in the greater activity of R22 and R63. The reduction in activity was in the order of 25 and 40% for Gin294 → Lys and Phe295 → Ala, respectively, and was shown to be statistically significant (p < 0.05) over 12 independent experiments. It is interesting to note that three of the four mutations that result in increased repression are changes to basic residues and that the changes cluster at amino acids 294–295 and 303–304. Although we cannot exclude the possibility that these amino acid changes act by enhancing the stability of the protein, the occurrence of gain of function mutants is somewhat surprising and suggests a possible relationship between the repression and co-activation functions of NGG1p.

The Amino-terminal 308 Amino Acids of NGG1p Contain an Activation Domain—NGG1p, as well as having a role in glucose repression, is required for transcriptional activation by a group of yeast regulators including LexA-GCN4p and GAL4-VP16 (27, 30, 32). Consistent with a role in activation, NGG1p can activate expression when tethered to DNA via a LexA DNA binding domain (31, 32). This activation is dependent on ADA2 (32). Furthermore, LexA-ADA2p activation requires NGG1 with this activity of NGG1p being partially fulfilled by the carboxy-terminal 339 amino acids of NGG1p but not the amino-terminal 346 residues (31, 32). To analyze the activation potential of the amino-terminal half of NGG1p when tethered to DNA as a GAL4 fusion, GAL4-NGG1p1–373 and point mutations thereof were introduced into yeast strain CY922, which also contained a his3-G4 lacZ fusion reporter. Trp+Leu+ (β-gal) transformants were grown in minimal medium containing 2% glucose, and β-galactosidase (β-gal) activity was determined after glass bead lysis of the cells using chlorophenol red-β-pro-p-galactosidase as substrate (51). The values shown are units of activity/μg of total protein.

| GAL4p derivative | β-Gal units |
|------------------|-------------|
| NGG1p1–373       | 270 ± 30    |
| NGG1p1–373 Phe295-Lys | 400 ± 50    |
| NGG1p1–373 Ala300-Pro | 260 ± 50    |
| NGG1p1–373 Gin294-Lys | 200 ± 30    |
| NGG1p1–373 Phe298-Ala | 70 ± 40     |
| p53              | 40 ± 8      |

Table II: Transcriptional activation by GAL4p-NGG1p1–373 derivatives

Transcriptional activation by GAL4p-NGG1p fusions. The coding sequence for amino acids 1–373 of NGG1p or derivatives thereof were cloned to the coding sequence for the DNA binding domain, amino acids 1–147, of GAL4p in the centromeric plasmid pAS1 (51). These GAL4-NGG1 fusions were transformed into yeast strain CY922 (gal4 gal80), which also contained a his3-G4 lacZ fusion reporter. Trp+Leu+ (β-gal) transformants were grown in minimal medium containing 2% glucose, and β-galactosidase (β-gal) activity was determined after glass bead lysis of the cells using chlorophenol red-β-pro-p-galactosidase as substrate (51). The values shown are units of activity/μg of total protein.

GAL4p-NGG1p, as well as having a role in glucose repression, is required for transcriptional activation by GAL4p-NGG1p1–373. The activation by GAL4p-NGG1p1–308 was comparable with that seen with GAL4p-NGG1p1–373.

The amino-terminal 308 amino acids of NGG1p contain an activation domain. This domain was affected by the same mutations that altered the glucose repression function of NGG1p. Alteration of Phe298 → Lys, the mutation that reduced glucose repression by approximately 2-fold, increased the strength of the activation domain. In contrast, the mutations that made NGG1p a more potent glucose repressor, Gin294 → Lys and Phe295 → Ala, reduced the transcriptional activation by GAL4p-NGG1p1–373. Ala300 → Pro, as was the case in glucose repression, had little effect on function. The most straightforward interpretation of the finding that mutations that affect glucose repression reciprocally alter activation by GAL4p-NGG1p1–373 is that the activation domain is influenced by or is part of the same repression domain that is involved with the glucose repression of GAL4p function. Furthermore, because the repression domain, as defined by the Phe298 → Lys, Phe295 → Ala, and Gin294 → Lys mutations, will act on the endogenous activation domain, these results strongly support the conclusion that the Phe-rich region has a direct role in transcriptional repression.

To further localize the activation domain, deletions of GAL4p-NGG1p1–373 were analyzed for their ability to activate the expression of an endogenous GAL-HIS3 fusion reporter in CY922. Activation by the GAL4p-NGG1p fusions can be determined by the relative growth rate of the strains on plates containing the competitive inhibitor of the his3 gene product AT. GAL4p-NGG1p1–373, in contrast to GAL4p-p53 (not shown), activates expression of GAL-HIS3 to a level that allows growth on plates containing 50 μM AT (Fig. 6). Deletion of amino acids 308–373 at the carboxy terminus of the fusion protein (GAL4p-NGG1p308) resulted in a marked enhancement of growth rate on plates containing 50 μM AT as compared with GAL4p-NGG1p1–373. The activation by GAL4p-NGG1p1–308 was comparable with that seen with GAL4p-NGG1pPhe298-Lys. These results indicate both that a principal
activation domain is amino-terminal to residue 308 and, based on the greater activation of the molecule lacking amino acids 308–373, that at least part of a repression domain is carboxy-terminal to this residue. The presence of a repression function in this region agrees with the finding that deletion of amino acids 308–373 also generates a molecule that is incapable of glucose repression (ngg1_339) deletion of the carboxyl terminus to residue 273 or beyond resulted in a loss of activation potential, thus placing at least part of the activation domain within the 274–307 region. The activation domain cannot, however, be simply ascribed to the 274–307 region, because deletions shorter than this range show that the region of NGG1p between amino acids 273 and 373 contains a repression domain that extends beyond amino acid 307 and second that an activation domain is positioned in part but not exclusively amino-terminal to amino acid 307. As might then be expected by the partial loss of both activation and repression functions, deletion of amino acids 274–307 results in only a slight overall change in activation potential of the fusion.

Horiiuchi and co-workers (31) have shown that the carboxyl terminus of NGG1p from amino acids 452 to 702 is able to interact with recombinant ADA2p in biochemical studies. If the amino-terminal 373 amino acids of NGG1p do not interact with ADA2p, then transcriptional activation by Gal4p–NGG1p1–373 may be independent of ADA2p. To test this possibility, Gal4p–NGG1p1–373 with the mutation of Phe298 → Lys and Gal4p–NGG1p1–373 were transformed into CY922 (ADA2) and CY936 (ada2) containing the his3-G25 lacZ reporter fusion, β-galactosidase activity of these strains was determined after growth in medium containing 2% glucose. As shown in Table III, transcriptional activation by Gal4p–NGG1p1–373 was reduced approximately 6-fold in the absence of ADA2p. Thus, although the amino-terminal 373 amino acids of NGG1p lack a domain shown to associate independently with ADA2p (32), transcriptional activation by this region still depends on ADA2p. The simplest interpretation would be that this region of NGG1p is capable of interacting either directly or indirectly with ADA2p. The incomplete activation of LexA-ADA2p by the carboxyl-terminal 339 amino acids of NGG1p seen by Horiiuchi et al. (32) may reflect the loss of this amino-terminal function. Surprisingly, the activity of Gal4p–NGG1p1–308 was not dependent on ADA2. In fact, a slight increase in transcriptional activation was seen in the ada2 strain CY936. If the region of amino acids 273–373 contains only one activation domain, then this domain does not require ADA2p for activity. This loss of a requirement for ADA2p with Gal4p–NGG1p1–308 also suggests that the region that provides the ADA2p dependence resides within amino acids 308–373.

ADA2p Is Required for Complete Glucose Repression of GAL10—If NGG1p acts exclusively in a complex with ADA2p, the latter protein may play a role in glucose repression. Glucose repression of the his3-G25 lacZ reporter was analyzed in strains containing single or double mutations of ngg1 and ada2 (Table IV). Disruption of ngg1 (CY914) resulted in an approximately 11-fold decrease in glucose repression of his3-G25 as compared with NGG1 (CY99). Disruption of ada2 (CY927) resulted in a 7.5-fold increase of glucose repression or approximately two-thirds of that seen in the ngg1 strain. The ngg1 ada2 double mutant strain, CY939, shows a response that is equivalent to the ngg1 strain. This effect is not likely the indirect result of ADA2p regulating the expression of NGG1 because an equivalent response was found in an ada2 background when NGG1 was expressed from the constitutive ded1 promoter (CY940). ADA2p, like NGG1p, is thus required for the complete glucose repression of his3-G25. Both NGG1p and ADA2p are likely to act in the same pathway because the phenotype of the double mutant is equivalent to the ngg1 strain. This along with the known association of ADA2p and NGG1p (32) suggests that the ADA complex is required for the glucose repression seen by NGG1p.
mentioned above, mutations can be made to the putative 17-amino acid helix that either positively or negatively affect glucose repression by NGG1p. Our mutational analyses define the importance of the presence and composition of the Phe-rich region to NGG1p function; however, the stability of the structure as suggested by the structural predictions makes it difficult to evaluate the role of its putative helical nature. Of the mutations analyzed, only the deletion mutants are sufficient to dramatically alter the structural predictions for this region. It should also be noted that as well as the Phe-rich region, ngg1Δ10 and ngg1Δ308–373, with deletions of amino acids 273–281 and 308–373, respectively, define at least two additional regions required for complete function of NGG1p in glucose repression. The proximity of these three sequences suggests that they may comprise part of a single functional domain.

The relationship of this Phe-rich region of NGG1p with those found in proteins such as HIV-gag and KEX1p is intriguing because there is no obvious functional relationship between these molecules. This lack of a functional relationship might suggest a structural role for the Phe-rich region; however, NGG1p derivatives lacking the Phe-rich region appeared as stable as the wild type molecule. Alternatively, the parallels between this amphipathic helix of NGG1p and those of Leu zipper-containing proteins (67) may suggest that the Phe-rich region may be involved in protein-protein interactions.

Glucose Repression by NGG1p—Probably Results from Its Action in an ADA Complex—NGG1p associates with at least two addition proteins, ADA2p and GCN5p. The carboxyl-terminal 250 amino acids of NGG1p interact with recombinant ADA2p in vitro, whereas the association of NGG1p and GCN5p is mediated through the latter’s interaction with ADA2p (31, 32). The coactivation properties of each of these three proteins is thought to occur by their action in a complex (27, 30–32). We have now shown that ADA2p, like NGG1p, is required for the complete glucose repression of the GAL10-related his3-G25 promoter. This supports the view that since similar to coactivation, NGG1p primarily exerts its action in glucose repression as part of an ADA complex. We interpret the finding that disruption of ngg1 has a more dramatic effect on the loss of glucose repression than ada2 to suggest that NGG1p is able to partially carry out the repression function independently or as part of a second complex.

Amino-terminal Activation Domain—We have mapped an activation domain in NGG1p in the amino-terminal half of the protein based upon its ability to activate transcription as a GAL4p fusion (GAL4p-NGG1p1–373). The analysis of the activation potential of deletion mutants indicates that the activation domain is found within amino acids 273–307 and thus is adjacent to or overlapping the region of NGG1p required for glucose repression. We believe that this activation domain is at least in part required for the coactivation function of NGG1p because deletions of this region are no longer capable of activating the expression of the PDR5 promoter.2

Horiiuchietal. (32) have previously shown that intact NGG1p will activate transcription when fused to LexA. Similar to this, the activation we have observed from the amino-terminal half of NGG1p is dependent on ADA2p. A functional relationship between NGG1p and ADA2p thus likely still exists in the absence of the carboxyl terminus of NGG1p. Although simplistic, an interaction between the amino terminus of NGG1p and ADA2p may stabilize the association seen for the carboxyl-terminal 250-amino acid region alone (32). The absence of this stabilization may account for the incomplete activation of LexA-ADA2p by the carboxyl-terminal 39 amino acids of NGG1p and for the inability of this fragment to function as a coactivator (32). Again it should be noted that the region of NGG1p from amino acids 308 to 373 is also critical for glucose repression.

The finding that GAL4p-NGG1p1–308 no longer requires ADA2p to act as a transcriptional activator is consistent with the region of amino acids 308–373 providing a functional link with ADA2p. Because the shorter fusion is more active than GAL4p-NGG1p1–250, ADA2p may act to regulate the transcriptional activation domain in NGG1p, perhaps by modulating its accessibility, rather than having a direct role in activation.

A Model for NGG1p Action—Several points must be considered in any model for the mechanism of glucose repression by NGG1p. 1) Based upon the similar phenotypes of ngg1 and ada2 in glucose repression of his3-G25, it is likely that NGG1p functions in glucose repression as part of an ADA complex. The individual functions of each of the members of the complex can thus likely be ascribed to the complex. 2) This same ADA complex or a related complex is required for transcriptional activation by a subset of activator proteins (27, 29, 30). 3) Interactions between ADA2p and transcriptional activator proteins allow the ADA complex to associate with activator proteins (34–36). In addition to the biochemical evidence showing this association, we have found that a direct or indirect association exists between NGG1p and GAL4p based on a two-hybrid analysis.2 This latter point agrees with the conclusion that GAL4p is the target for NGG1p action in glucose repression of the GAL genes. 4) ADA2p has been found to associate with TBP on affinity columns (35). 5) NGG1p contains an internal domain that is essential for glucose repression. This domain is proximal to or overlapping a domain that activates transcription when fused to the DNA binding domain of GAL4p.

In the simplest case we envisage a model for repression in which the ADA complex associates with GAL4p in an interaction mediated by ADA2p and perhaps other components of the ADA complex. Because the complex can either stimulate or repress transcription depending on the activator protein in question, it probably also associates with a second component required for transcriptional activation such as one of the basal factors, another coactivator, or a chromatin component. The association of ADA2p from crude yeast extracts with TBP on affinity columns suggests that TBP is the likely target for the complex (35). It is likely that this association accounts for the transcriptional activation by ADA complex proteins when tethered directly to DNA. To this point the model is fully consistent with those proposed for the relief of VP16 toxicity by the ada mutations in their original identification (27, 29). The fact that the Phe-rich region is essential for both transcriptional activation by GAL4p-NGG1p fusions and transcriptional repression by NGG1p supports the idea that these activities are mechanistically related and perhaps that the Phe-rich region is directly or indirectly involved in the interaction with the downstream effector. Repression of transcription by NGG1p could result from its inhibiting the productive activity of the downstream factor similar to the mechanism of inhibition exerted by the negative factors DR1 (NC2) (68, 69) and MOT1p (70–72) on TBP. In a unified model for coactivation and repression, NGG1p may either stimulate or repress the activity of the downstream effector depending upon conformational changes that are influenced by the nature of its interaction with the activator. Perhaps it is by subtly altering a balance between activation and repression that certain NGG1p derivatives can act as better repressors. Analyses can be made with models proposed for the human thyroid hormone receptor-β, which switches from a repressor to an activator upon binding thyroid.

2 J. A. Martens and C. J. Brandl, unpublished data.
hormone (73). Both forms of the receptor bind TFIIIB, but the result of the interaction, transcriptional activation or repression, depends on the conformational state of the receptor. In the case of NGG1p, the conformational state may be determined by the activator.

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Structure/Function Properties of the Yeast Dual Regulator Protein NGG1 That Are Required for Glucose Repression

Christopher J. Brandl, Joseph A. Martens, Adit Margaliot, David Stenning, Angela M. Furlanetto, Ayman Saleh, Katherine S. Hamilton and Julie Genereaux

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