

How the Rgt1 Transcription Factor of *Saccharomyces cerevisiae* Is Regulated by Glucose

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ABSTRACT

Rgt1 is a transcription factor that regulates expression of *HXT* genes encoding glucose transporters in the yeast *Saccharomyces cerevisiae*. Rgt1 represses *HXT* gene expression in the absence of glucose; high levels of glucose cause Rgt1 to activate expression of *HXT1*. We identified four functional domains of Rgt1. A domain required for transcriptional repression (amino acids 210–250) is required for interaction of Rgt1 with the Ssn6 corepressor. Another region of Rgt1 (320–380) is required for normal transcriptional activation, and sequences flanking this region (310–320 and 400–410) regulate this function. A central region (520–830) and a short sequence adjacent to the zinc cluster DNA-binding domain (80–90) inhibit transcriptional repression when glucose is present. We found that this middle region of Rgt1 physically interacts with the N-terminal portion of the protein that includes the DNA-binding domain. This interaction is inhibited by the Rgt1 regulator Mth1, which binds to Rgt1. Our results suggest that Mth1 promotes transcriptional repression by Rgt1 by binding to it and preventing the intramolecular interaction, probably by preventing phosphorylation of Rgt1, thereby enabling Rgt1 to bind to DNA. Glucose induces *HXT1* gene expression by causing Mth1 degradation, allowing Rgt1 phosphorylation, and leading to the intramolecular interaction that inhibits DNA binding of Rgt1.

THE yeast *Saccharomyces cerevisiae* prefers to fuel its growth with glucose and regulates gene expression accordingly. Glucose turns off expression of many genes not required for growth on glucose and induces expression of other genes that participate in glucose utilization (JOHNSTON 1999; OZCAN and JOHNSTON 1999; ROLLAND *et al.* 2002; GELADE *et al.* 2003). Glucose induces expression of several *HXT* genes that encode glucose transporters, which facilitate the first step of glucose utilization (KO *et al.* 1993). Glucose induces *HXT* gene expression through a signal transduction pathway that begins at the cell surface with glucose sensors and culminates in the nucleus with alteration of function of the Rgt1 transcription factor, which binds to the promoters of several *HXT* genes and regulates their expression (JOHNSTON 1999; GELADE *et al.* 2003).

Rgt1 represses transcription in the absence of glucose. This requires the corepressors Ssn6 and Tup1 (OZCAN and JOHNSTON 1995; YANG and BISSON 1996; TOMASCOBOS and SANZ 2002) and at least one of the paralogous proteins Mth1 and Std1 (SCHMIDT *et al.* 1999; LAKSHMANAN *et al.* 2003; MOSLEY *et al.* 2003). Mth1 is required for the DNA-binding ability of Rgt1 (FLICK *et al.* 2003), possibly because it inhibits the glucose-induced phosphorylation of Rgt1 (KIM *et al.* 2003; LAKSHMANAN *et al.* 2003; MOSLEY *et al.* 2003). Glucose inhibits Rgt1

function by stimulating degradation of Mth1 (FLICK *et al.* 2003; MORIYA and JOHNSTON 2004), which leads to hyperphosphorylation of Rgt1 and loss of its DNA-binding activity. Std1, which is also degraded in response to glucose, does not seem to regulate the DNA-binding activity of Rgt1.

When glucose levels are high (>2%), Rgt1 activates transcription (OZCAN *et al.* 1996). This conclusion derives from the observations that LexA-Rgt1 activates expression of a gene containing a LexA-binding site, and deletion of *RGT1* reduces the level of glucose induction of *HXT1* expression (OZCAN and JOHNSTON 1995). No model for regulation of Rgt1 function has been put forward.

Rgt1 is one of only a few transcription factors in yeast known to have two different effects on transcription. Ume6 recruits Sin3-Rpd3 to direct repression of some early meiotic genes through histone deacetylase interactions (KADOSH and STRUHL 1997; KADOSH and STRUHL 1998; WASHBURN and ESPOSITO 2001), and it recruits Ime1 to activate transcription of other early meiotic genes (GUO and KOHLHAW 1996; RUBIN-BEJERANO *et al.* 1996; KASSIR *et al.* 2003). Leu3 is regulated without the help of other proteins (GUO and KOHLHAW 1996): in the presence of the ligand, α -isopropylmalate, Leu3 is a transcriptional activator, but in its absence regions of Leu3 mask the transcriptional activation domain and cause it to repress transcription through a separate repression domain (WANG *et al.* 1997, 1999).

Here we describe the results of experiments that iden-

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TABLE 1
Yeast strains

Strain	Genotype	References
YM 4509	<i>MATa ura3-52 his3Δ200 ade2-101 lys2-801 leu2 trp1-903 tyr1-501 GAL4 GAL80 rgt1Δ::hisG</i>	OZCAN <i>et al.</i> (1996)
YM 6150	<i>MATa ura3-52 his3Δ200 ade2-101 lys2-801 leu2 trp1-903 tyr1-501 GAL4 GAL80 rgt1Δ::hisG HXT3-HIS3::TRP1</i>	This study
YM 6243	<i>MATa ura3-52 his3Δ200 ade2-101 lys2-801 leu2 trp1-903 tyr1-501 GAL4 GAL80 rgt1Δ::hisG HXT3-HIS3::TRP1 HXT1-lacZ::URA3</i>	This study
YM 6800	<i>MATa ura3-52 his3Δ200 ade2-101 lys2-801 leu2 trp1-903 tyr1-501 GAL4 GAL80 rgt1Δ::hisG 8xlexO-lacZ::URA3</i>	This study
FM 353	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80D GAL2-ADE2 lys2::GAL1-HIS3 met2::GAL7-lacZ</i>	JAMES <i>et al.</i> (1996)

tify four functional domains of Rgt1. One is involved in transcriptional repression, one appears to be involved in transcriptional activation, and two domains regulate these functions. We identified regions of Rgt1 responsible for its interactions with Mth1, Std1, and Ssn6. Our results lead us to suggest a model for regulation of Rgt1 function that involves intra- and intermolecular protein interactions that respond to glucose availability.

MATERIALS AND METHODS

Construction of Rgt1 mutations: Deletions of 30 codons of *RGT1* were made by “gap repair” of a plasmid using two *RGT1* PCR products (Figure 1). Using a plasmid carrying *lexA-RGT1* (BM 3306) as a template, one PCR product is generated from the 5' end of *RGT1* using a universal “forward” primer (OM 1620) that anneals to plasmid sequences that are also present in the plasmid recipient of the gap repair. The “reverse” primer (A in Figure 1) anneals to the region of *RGT1* directly upstream of the 10 codons to be deleted. The 18 3' nucleotides of this primer correspond to six histidine codons. The other PCR product used for the gap repair is generated using a “forward” primer (B in Figure 1) that anneals to the region of *RGT1* directly downstream of the codons to be deleted and has 18 nucleotides corresponding to six histidine codons at its 3' end. The “reverse” primer (OM 3083) is universal and anneals to sequences in the template as well as the recipient plasmids. Approximately 0.5 μg of the two PCR products is combined with ~10 ng of a CEN-*LEU2* plasmid (pRS315; SIKORSKI and HIETER 1989) linearized by digestion with *SalI* and used to transform yeast cells to Leu⁺ (YM 4509, deleted for *RGT1* to avoid recombination of the transforming DNA with the genome; Table 1). Each deletion mutation was verified by observing the expected DNA fragments following digestion with *SalI* and *EcoRI*.

A similar approach was used to change single nucleotides of *RGT1*. Two oligonucleotides, both containing complementary single-nucleotide changes that will result in a point mutation, are used as primers (analogous to A and B in Figure 1) with the universal primers (OM 1620 and OM 3083) to amplify in separate reactions the 5' and 3' portions of *RGT1*, using pBM 3580 as template. The products, (which overlap by the length of the PCR primers) are combined with the empty vector (BM 3564) linearized by digestion with *SalI* and used to transform yeast (YM 4509) to Leu⁺.

β-Galactosidase assays: β-Galactosidase activity assays were performed using the yeast β-galactosidase assay kit (Pierce,

Rockford, IL) following the manufacturer's instructions. Results are presented in Miller units [$1000 \times \text{OD}_{420}$ (ONPG)/ OD_{600} (cells) \times vol (ml) \times time (min)]. The activity reported is the average of assays performed in duplicate on four independent transformants. Cells were grown to log phase (OD_{600} 1–2) in synthetic media containing 2% galactose and 0.05% glucose and then transferred to synthetic media containing the appropriate carbon source [2% galactose (repressing conditions) and/or 2% glucose (activating conditions)], and incubated at 30° for 4 hr before assaying β-galactosidase.

Detecting interactions of Mth1, Std1, and Ssn6 with Rgt1 *in vitro*: A total of 500 ml of *Escherichia coli* cells (DH5α) containing plasmids expressing GST (BM 4266), GST-TPR (BM 4256, gift from Dimitris Tzamarias), GST-Std1 (BM 3923), or GST-Mth1 (BM 4344) were grown to log phase in LB-Amp media. IPTG (0.25 mM) was added 3 hr before harvesting the cells by centrifugation. Cells were resuspended in 5 ml of buffer [100 mM NaCl, 20 mM HEPES at pH 7.5, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 20% glycerol, 0.5% BSA, and complete, EDTA-free protease inhibitors (Roche)] and sonicated 10 times for 10 sec followed by centrifugation at $10,000 \times g$ for 15 min. The supernatant was incubated with an equal volume (0.5–2.0 ml) of agarose beads linked to glutathione (Sigma, St. Louis) for 1 hr at 4°. The beads were then washed four times with 10 volumes of the same buffer minus BSA and stored at 4° (TZAMARIAS and STRUHL 1995). ³⁵S-labeled Rgt1 was synthesized *in vitro* using TNT T7 Quick for PCR DNA (Promega, Madison, WI) according to the manufacturer's instructions. In total, 10 μl of this reaction was incubated with 1–2 μg of agarose beads carrying the GST fusion protein in 400 μl buffer containing 100 mM NaCl, 20 mM Tris-HCl at pH 8.0, 0.1% NP-40, and 0.25% BSA plus complete, EDTA-free protease inhibitors (Roche) for 2 hr at 4° with rocking. The beads were washed three times with 1.5 ml of the same buffer and once with the same buffer lacking BSA. The bound protein was eluted in SDS sample buffer and subjected to SDS-PAGE. The gel was dried and exposed overnight to film (TZAMARIAS and STRUHL 1995).

GST-Mth1 pull-downs using LexA-Rgt1 derivatives from cell lysates: Yeast cell extracts were prepared from cells expressing LexA-Rgt1 by vortexing the cell pellets with glass beads in NP-40 buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 1% NP-40) at 4° for 10 min and then 3 mg of crude protein was incubated at 4° for 3 hr with agarose beads conjugated to anti-LexA (Santa Cruz Biotechnology). The beads were then washed with high-salt buffer (NP-40 containing 1 M NaCl) and resuspended in the binding buffer (25 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 0.1% Tween20). GST-Mth1 purified from *E. coli* cell extract was mixed with the beads and incubated

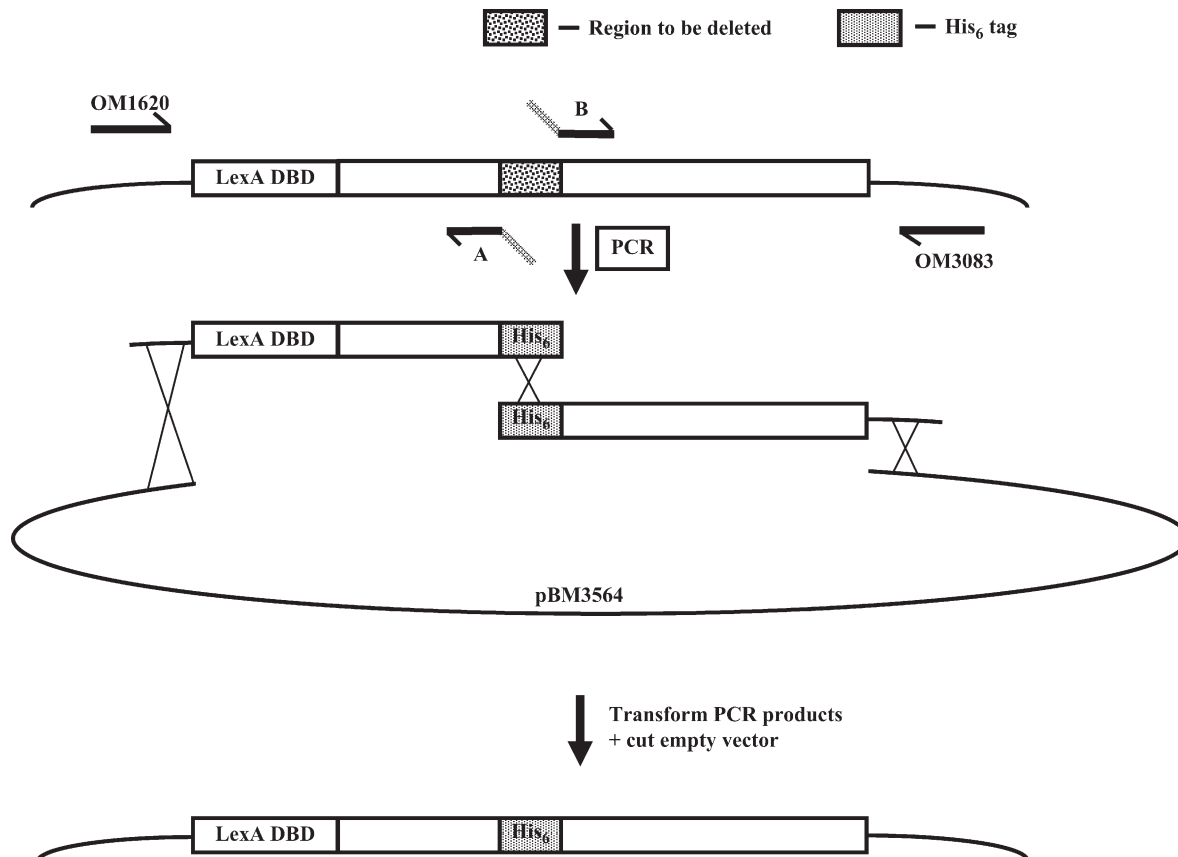


FIGURE 1.—Scheme for generating 10-codon deletion/His₆ insertion mutations in *RGT1*. *RGT1* sequences flanking the region to be deleted are amplified by the PCR using primers with tails of 6 His codons (stippled). The PCR products are used to “gap repair” a linearized plasmid (pBM 3564 cleaved with *SalI*) by transformation of yeast to Leu⁺. The resulting *RGT1* mutation replaces 10 codons with 6 His codons. See MATERIALS AND METHODS for details.

2 hr at room temperature. The beads were washed with five volumes of the binding buffer and proteins were resolved on a 7.5% SDS gel and detected with anti-LexA and GST antibodies (Santa Cruz) after blotting to filter paper.

Immunoprecipitations: Extracts of yeast cells expressing LexA-Rgt1 fusion proteins from the *ADHI* promoter were prepared by vortexing cells with acid-washed glass beads (0.5-mm diameter) in NP-40 lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 1% NP-40) containing phosphatase inhibitors (10 mM Na-pyrophosphate, 200 μ M Na-orthovanadate, and 50 mM Na-fluoride) at 4° for 10 min. The cell lysates (2 mg) were incubated with anti-LexA mouse monoclonal antibody (Santa Cruz) at 4° for 3 hr and further incubated with protein G-conjugated agarose beads (Santa Cruz) for 1 hr. After the beads were washed with NP-40 lysis buffer containing 1 M NaCl, proteins were eluted by boiling in SDS sample buffer for 5 min and were resolved in SDS-polyacrylamide gels.

SDS-PAGE and Western blot analysis: Cells carrying plasmids encoding a LexA-Rgt1 chimera with mutations were grown in minimal media to an OD₆₀₀ of 2.5, harvested by centrifugation, and suspended in 100 μ l of water. An equal volume of 0.2 M NaOH was added, and the cells were incubated for 5 min at room temperature. The cells were harvested by centrifugation, resuspended in 50 μ l SDS-sample buffer (62.5 mM Tris-HCl at pH 6.8, 25% glycerol, 2% SDS, and 0.01% bromophenol blue), and boiled for 5 min; 10 μ l was then loaded on SDS-PAGE (LAEMMLI 1970; KUSHNIROV 2000). Gels were transferred to a PVDF membrane (Millipore, Bedford,

MA) and probed with goat anti-mouse LexA monoclonal antibody (Santa Cruz).

Chromatin immunoprecipitation: Rgt1 binding to the *HXT1* promoter *in vivo* was assayed by chromatin immunoprecipitation as described previously (Kim *et al.* 2003). Genomic DNA fragments crosslinked to Rgt1 were immunoprecipitated with anti-Rgt1 antibody and Protein A-agarose beads (Santa Cruz). The DNA sequence upstream of *HXT* genes in the immunoprecipitate was amplified by the PCR using a primer pair, OM 2642 and OM 2643. Sequences of the primers are available on request.

RESULTS

Functional domains of Rgt1: We scanned Rgt1 for functional domains by deleting successive 10-amino-acid segments and scoring the resulting proteins for their ability to repress or activate gene expression. The deletions were constructed by “gap repair” of a plasmid with DNA fragments that flank the deletion, generated by the PCR (see Figure 1 and MATERIALS AND METHODS for details). In total, 108 10-amino-acid deletions were created starting just C-terminal to the Rgt1 zinc finger (Δ 80–90) and extending to the C terminus (Δ 1160–1170). [The region upstream of the zinc finger was ignored because Rgt1 missing these sequences (Rgt1 Δ 1-

TABLE 2
Phenotypes associated with 108 10-amino-acid deletions/His₆ insertions in Rgt1

Rgt1 ^a	Fold repression on 2% Gal ^b	Fold activation on 2% Glu ^c
wt	10.0	30.0
Δ80–90	15.7 ^d	0.08 (12.5-fold repression) ^d
Δ90–100	5.3	48.6
Δ100–110	3.8	28.2
Δ110–120	9.4	32.4
Δ120–130	10.9	41.0
Δ130–140	19.3	28.6
Δ140–150	7.8	37.9
Δ150–160	2.4	53.1
Δ160–170	9.8	35.8
Δ170–180	8.7	44.4
Δ180–190	6.8	29.5
Δ190–200	7.9	33.9
Δ200–210	10.3	32.7
Δ210–220	1.1 ^e	23.5 ^e
Δ220–230	1.2 ^e	28.4 ^e
Δ230–240	1.0 ^e	32.8 ^e
Δ240–250	1.0 ^e	18.7 ^e
Δ250–260	12.2	25.5
Δ260–270	6.8	18.2
Δ270–280	15.6	28.3
Δ280–290	5.7	18.2
Δ290–300	3.0	16.1
Δ300–310	3.0	29.5
Δ310–320	0.04 (26-fold activation) ^f	51.5 ^f
Δ320–330	10.5 ^g	2.7 ^g
Δ340–350	8.5 ^g	8.7 ^g
Δ350–360	14.2 ^g	5.3 ^g
Δ360–370	12.5	21.8
Δ370–380	9.8 ^g	6.5 ^g
Δ380–390	10.2	28.8
Δ390–400	12.1	31.1
Δ400–410	0.04 (24-fold activation) ^f	21.9 ^f
Δ410–420	9.5	26.6
Δ420–430	8.4	38.2
Δ430–440	6.3	25.2
Δ460–470	13.5	37.1
Δ470–480	15.6	28.9
Δ480–490	22.8	22.8
Δ490–500	31.6	17.4
Δ500–510	35.9	15.7
Δ510–520	49.3	20.4
Δ520–530	4.1 ^d	0.24 (4.2-fold repression) ^d
Δ530–540	5.5 ^d	0.33 (3.0-fold repression) ^d
Δ540–550	3.2 ^d	0.24 (4.2-fold repression) ^d
Δ550–560	4.6	10.1
Δ560–570	5.2	12.8
Δ570–580	7.7	10.0
Δ580–590	6.8	13.4
Δ590–600	4.2	17.1
Δ600–610	4.8	25.1
Δ610–620	8.6	21.7
Δ620–630	4.3	28.7

(continued)

TABLE 2
(Continued)

Rgt1 ^a	Fold repression on 2% Gal ^b	Fold activation on 2% Glu ^c
Δ630–640	3.7	17.2
Δ640–650	4.0 ^d	0.25 (4.0-fold repression) ^d
Δ650–660	4.5 ^d	0.22 (4.5-fold repression) ^d
Δ660–670	3.5 ^d	0.31 (3.2-fold repression) ^d
Δ670–680	5.3 ^d	0.19 (5.2-fold repression) ^d
Δ680–690	4.1 ^d	0.23 (4.3-fold repression) ^d
Δ690–700	5.4 ^d	0.36 (2.8-fold repression) ^d
Δ700–710	11.0 ^d	0.31 (3.2-fold repression) ^d
Δ710–720	3.4 ^d	0.34 (2.9-fold repression) ^d
Δ720–730	6.9	22.1
Δ750–760	3.2 ^d	0.24 (4.2-fold repression) ^d
Δ760–770	3.8 ^d	0.18 (5.6-fold repression) ^d
Δ770–780	3.4 ^d	0.17 (5.9-fold repression) ^d
Δ780–790	3.4 ^d	0.25 (4.0-fold repression) ^d
Δ790–800	6.3 ^d	0.26 (3.8-fold repression) ^d
Δ810–820	4.7 ^d	0.14 (7.1-fold repression) ^d
Δ820–830	5.4 ^d	0.13 (7.7-fold repression) ^d
Δ830–840	6.3	15.4
Δ840–850	3.7	11.6
Δ850–860	5.8	23.3
Δ860–870	11.8	25.1
Δ870–880	21.4	15.5
Δ900–910	5.6	14.0
Δ910–920	5.0	21.1
Δ970–980	5.9	15.2
Δ980–990	5.6	21.2
Δ990–1000	7.8	18.1
Δ1040–1050	4.6	25.4
Δ1050–1060	3.0	15.6
Δ1080–1090	3.3	18.1
Δ1090–1100	3.6	12.1
Δ1100–1110	8.9	21.5
Δ1110–1120	5.9	12.9
Δ1130–1140	10.5	15.5
Δ1140–1150	4.1	19.6
Δ1150–1160	11.4	14.9

Plasmids with the *rgt1* mutations were introduced into *rgt1Δ* cells (YM 4509) containing the *HXT1-lacZ* reporter (pBM 2637) and the *lexO-lacZ* reporter (pBM 1817) to assay transcriptional repression and transcriptional activation, respectively. Duplicates of four independent cultures were grown (see MATERIALS AND METHODS for growth conditions) and assayed for their β-galactosidase activity. The average of eight assays was determined and activity was calculated relative to a strain

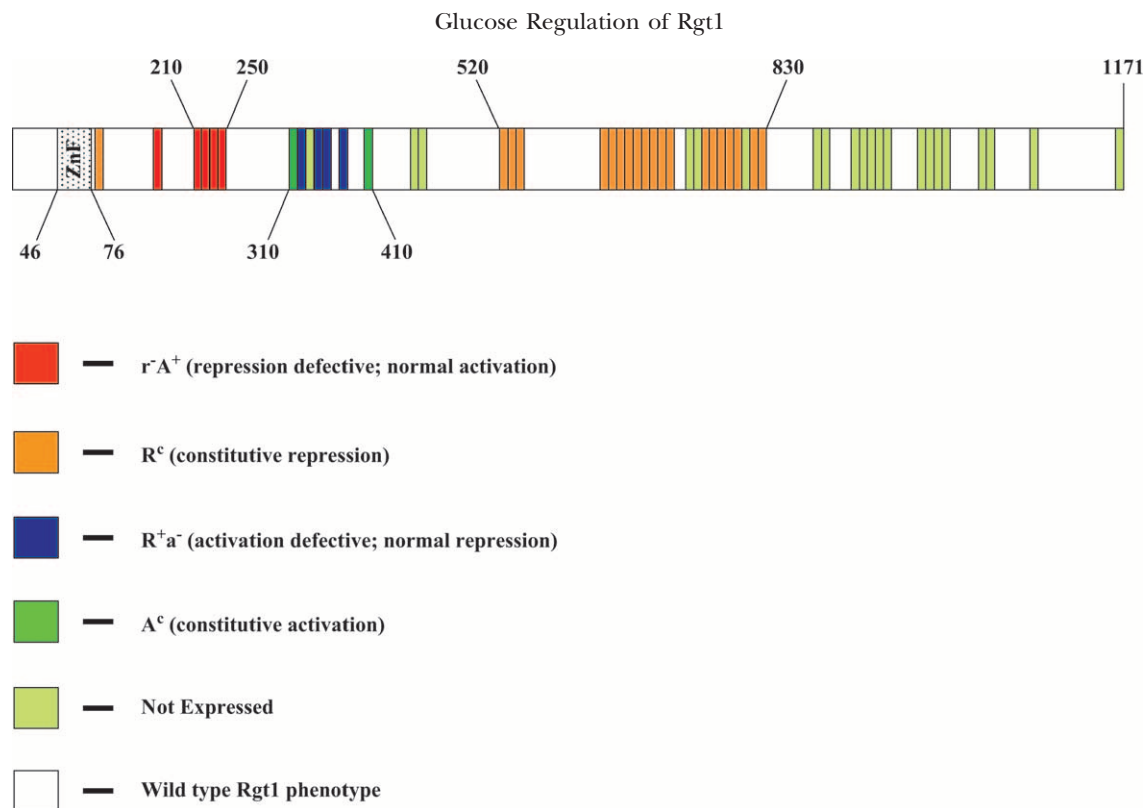


FIGURE 2.—Summary of the results of mutational analysis of Rgt1 function. The 10-amino-acid deletions begin just downstream of the zinc finger (stippled box). The first deletion is $\Delta 80-90$ and the last deletion is $\Delta 1160-1170$. Only those deletions that cause a phenotype or prevent expression of Rgt1 are represented, color-coded to the phenotype. The deletions of *RGT1* that cause loss of transcriptional repression [but do not affect transcriptional activation in glucose-grown cells (r^-A^+)] are shown in red; deletions that cause constitutive repression [*i.e.*, repression of gene expression in cells grown in the presence of glucose as well as galactose (R^c)] are depicted in orange. Deletions that reduce transcriptional activation [but still promote normal transcriptional repression in galactose-grown cells (R^+a^-)] are shown in blue. Deletions that cause constitutive transcriptional activation [*i.e.*, activation of gene expression in cells grown on galactose as well as glucose (A^c)] are shown in green.

75) appears to be regulated normally (OZCAN *et al.* 1996)].

The ability of the altered Rgt1 proteins to repress transcription in galactose-grown cells was assessed using an *HXT1-lacZ* reporter (pBM 2637); their ability to activate transcription in glucose-grown cells was assessed with a *lexO-lacZ* reporter (pBM 1817; the LexA DNA-

binding domain is fused to the N terminus of all constructs; see MATERIALS AND METHODS for details). The results of this analysis are presented in Table 2 and summarized in Figure 2. Four different phenotypes are apparent among the deletion mutants. First, transcriptional repression in galactose-grown cells is abolished when amino acids 210–250 are deleted, suggesting that

TABLE 2
(Continued)

harboring LexA alone (*i.e.*, with no Rgt1 fusion). Repression in a mutant is considered to be deficient if β -galactosidase activity in galactose-grown cells is more than one-third of that in the LexA control strain (*i.e.*, <3 -fold repression; cells with wild-type *RGT1* have 1/10 of the β -galactosidase activity of cells with no *RGT1* (LexA only) under these conditions, *i.e.*, 10-fold repression).

^a Level of Rgt1 in cells was assessed by Western blotting using an antibody to LexA_{DBD} to detect Rgt1. All proteins listed in the table were present at least at $\sim 5\%$ of the level of Rgt1 in wild-type cells (but usually much more); *rgt1* deletions that caused Rgt1 to be undetectable were excluded from this analysis.

^b Mutations are classified as repression deficient if they effect <3 -fold transcriptional repression.

^c Mutations are classified as activation deficient if they effect <10 -fold transcriptional activation.

^d Constitutive repressor alleles (*i.e.*, repressing in glucose-grown as well as galactose-grown cells). Transcriptional activation is considered to be deficient if β -galactosidase activity in glucose-grown cells is <10 -fold over the LexA control strain (*i.e.*, <10 -fold activation; cells with wild-type *RGT1* have 30 times the β -galactosidase activity of cells with no *RGT1* (LexA only) under these conditions, *i.e.*, 30-fold activation).

^e Mutants deficient in repression.

^f Constitutive activation alleles (*i.e.*, activating in galactose-grown as well as glucose-grown cells).

^g Activation-deficient alleles.

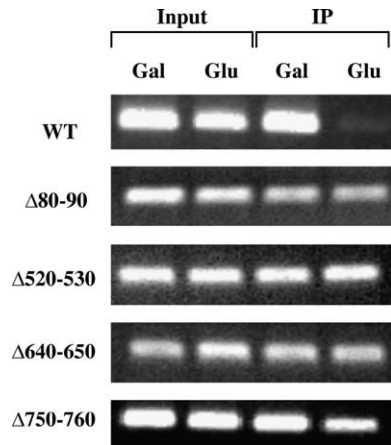


FIGURE 3.—Rgt1 constitutive repressors bind constitutively to the *HXT1* promoter. Chromatin was prepared from wild-type yeast cells containing *lexA*-Rgt1 fusions: wild type (pBM 3580), $\Delta 80-90$ (pBM 3976), $\Delta 520-530$ (pBM 3970), $\Delta 640-650$ (pBM 3972), and $\Delta 750-760$ (pBM 4058). The cells were grown on YP media containing different carbon sources as indicated above each lane and their chromatin was immunoprecipitated using anti-*lexA* antibody. The *HXT1* promoter in the immunoprecipitated DNA (IP) was detected by ethidium bromide staining after amplifying it in a PCR and resolving it by electrophoresis through a 2% agarose gel.

these residues encompass a transcriptional repression domain. Second, deletions of amino acids 320–380 impair transcriptional activation by Rgt1 in glucose-grown cells, suggesting that this region of the protein contributes to transcriptional activation. Third, many of the deletions of amino acids 520–830, as well as deletion of residues 80–90, cause Rgt1 to be a constitutive repressor, inhibiting gene expression in cells grown on 2% glucose as well as in cells grown on media containing 2% galactose. Indeed, Rgt1 proteins missing portions of this region bind DNA constitutively (Figure 3). Thus, these

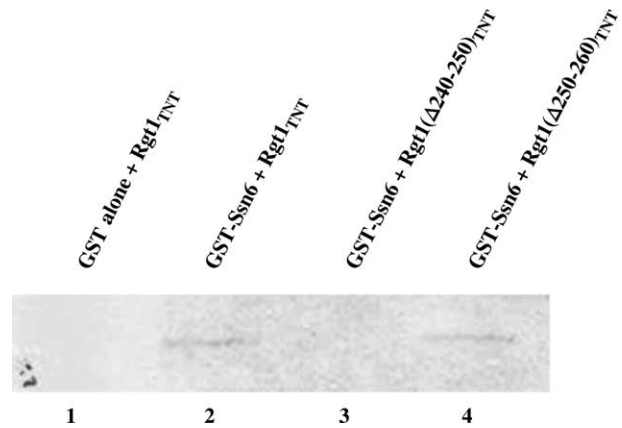


FIGURE 4.—*In vitro* interaction between Rgt1 and Ssn6. Agarose beads conjugated to glutathione were incubated with GST-Ssn6 (produced in *E. coli* from pBM 4266) and 20% of the ^{35}S -Met-labeled Rgt1 produced by *in vitro* transcription/translation (TNT) of a PCR-generated *RGT1* ORF with a T7 promoter. The beads were incubated at 4° for 2 hr and washed four times with wash buffer as described in MATERIALS AND METHODS. Proteins were eluted from the beads with Laemmli buffer containing 5% β -mercaptoethanol, separated by SDS-PAGE, transferred to PVDF membrane, and exposed to film for at least 24 hr. Lane 1, GST alone (pBM 4266) incubated with Rgt1_{TNT}; lane 2, GST-Ssn6 incubated with Rgt1_{TNT}; lane 3, GST-Ssn6 incubated with Rgt1 $\Delta 240-250$ _{TNT}; lane 4, GST-Ssn6 incubated with Rgt1 $\Delta 250-260$ _{TNT}. Approximately 50% of the total protein added was retained by the fusion proteins in lanes 2 and 4 (input not shown).

regions of Rgt1 seem to operate to inhibit Rgt1 repressor function when glucose is available. Finally, two deletions ($\Delta 310-320$ and $\Delta 400-410$) convert Rgt1 into a constitutive activator (*i.e.*, it activates transcription even in the absence of glucose), suggesting that these parts of Rgt1 inhibit transcriptional activation when glucose is absent. (Note that a number of deletions prevented stable ex-

TABLE 3

Two-hybrid analysis of the Ssn6-Rgt1 interaction

Line	DNA-binding hybrid ^a	Activation hybrid ^b	<i>lexO-lacZ</i> expression
1	Rgt1	Empty vector	5 ± 1.4
2	Empty vector	Ssn6	2 ± 0.7
3	Rgt1	Ssn6	229 ± 16.3
4	Rgt1 ($\Delta 190-200$)	Ssn6	206 ± 20.6
5	Rgt1 ($\Delta 200-210$)	Ssn6	50 ± 7.4
6	Rgt1 ($\Delta 210-220$)	Ssn6	5 ± 0.6
7	Rgt1 ($\Delta 220-230$)	Ssn6	3 ± 0.3
8	Rgt1 ($\Delta 230-240$)	Ssn6	7 ± 1.6
9	Rgt1 ($\Delta 240-250$)	Ssn6	5 ± 0.9
10	Rgt1 ($\Delta 250-260$)	Ssn6	210 ± 23.5

Cells carrying the *8lexO-lacZ* reporter (pBM 4041) containing AD-SSN6 (pBM 3232) and various portions of *RGT1* fused to *lexA* were grown in medium containing 2% galactose (conditions that cause Rgt1 to repress transcription). Since Rgt1 activates transcription in the presence of glucose, it is impossible to assess the Rgt1-Ssn6 interaction by this method with cells grown in 2% glucose.

^a Rgt1 fused to LexA_{BD} in pSH2-1 (BRENT and PTASHNE 1985; MA and PTASHNE 1987b).

^b Ssn6 fused to the Gal4 transcriptional activation domain in pOAD (BARTEL *et al.* 1996).

TABLE 4
Two-hybrid analysis of Rgt1-Mth1/Std1 interaction

Line	Rgt1-AD ^a	Std1-BD ^b	Mth1-BD ^b
1	Empty vector	1.2 ± 0.8	0.3 ± 0.2
2	Rgt1	63.0 ± 4.5	91.5 ± 12.6
3	Rgt1 (Δ300-310)	45.8 ± 6.5	87.3 ± 10.3
4	Rgt1 (Δ310-320)	6.7 ± 1.4	98.4 ± 11.4
5	Rgt1 (Δ320-330)	50.2 ± 7.5	74.3 ± 8.2
6	Rgt1 (Δ340-350)	68.1 ± 4.1	80.2 ± 7.6
7	Rgt1 (Δ350-360)	56.4 ± 3.5	10.3 ± 1.2
8	Rgt1 (Δ360-370)	67.2 ± 10.5	78.8 ± 9.1
9	Rgt1 (Δ370-380)	68.2 ± 7.1	54.1 ± 6.3
10	Rgt1 (Δ380-390)	64.8 ± 6.9	40.3 ± 3.2
11	Rgt1 (Δ390-400)	60.1 ± 5.6	73.3 ± 5.4
12	Rgt1 (Δ400-410)	58.4 ± 6.7	61.1 ± 7.1

^aRgt1 fused to the Gal4 transcriptional activation domain in pOAD (BARTEL *et al.* 1996).

^bStd1 and Mth1 fused to the Gal4 DNA-binding domain in pOBD (BARTEL *et al.* 1996).

pression of Rgt1 and therefore could not be assayed for their effect on Rgt1 function).

Rgt1 recruits Ssn6, Mth1, and Std1: Because the Ssn6-Tup1 corepressor complex is known to be recruited by other DNA-binding proteins via Ssn6 (KELEHER *et al.* 1989; BALASUBRAMANIAN *et al.* 1993; TREITEL and CARLSON 1995; PARK *et al.* 1999; SMITH and JOHNSON 2000),

we tested for interaction between Ssn6 and Rgt1 using the two-hybrid method. Ssn6 clearly interacts with Rgt1 in galactose-grown cells, and this requires the repression domain of Rgt1 (amino acids 210–250; Table 3). Amino acids immediately adjacent to the repression domain (190–200 and 250–260) are not required for the interaction of Rgt1 with Ssn6. The Rgt1-Ssn6 interaction was confirmed *in vitro*: full-length Rgt1 expressed in a cell-free system was retained on a glutathione-agarose column containing GST-Ssn6 produced in *E. coli* (Figure 4, lanes 2 and 4). The interaction requires Rgt1 amino acids 240–250 (lane 3), which lie in the putative repression domain, but not 250–260, which lie immediately adjacent to this functional domain (lane 4).

Mth1 and Std1 are negative regulators of the transcriptional activation function of Rgt1 (OZCAN *et al.* 1993; SCHMIDT *et al.* 1999) that also interact with Rgt1 (TOMAS-COBOS and SANZ 2002; LAKSHMANAN *et al.* 2003). We verified these interactions with the two-hybrid method and found that Rgt1 amino acids 310–320 are required for its interaction with Std1, and amino acids 350–360 are required for maximal interaction with Mth1 (Table 4). Mth1 and Std1 interact with Rgt1 in galactose-grown cells (Table 4), but not in cells grown on 2% glucose (data not shown). Std1 and Mth1 interact *in vitro* with full-length Rgt1 (Figure 5A, lane 3, and 5B, lane 2, respectively). In addition, both Std1 and Mth1 interact with the first third of Rgt1 (Figure 5A, lane 5,

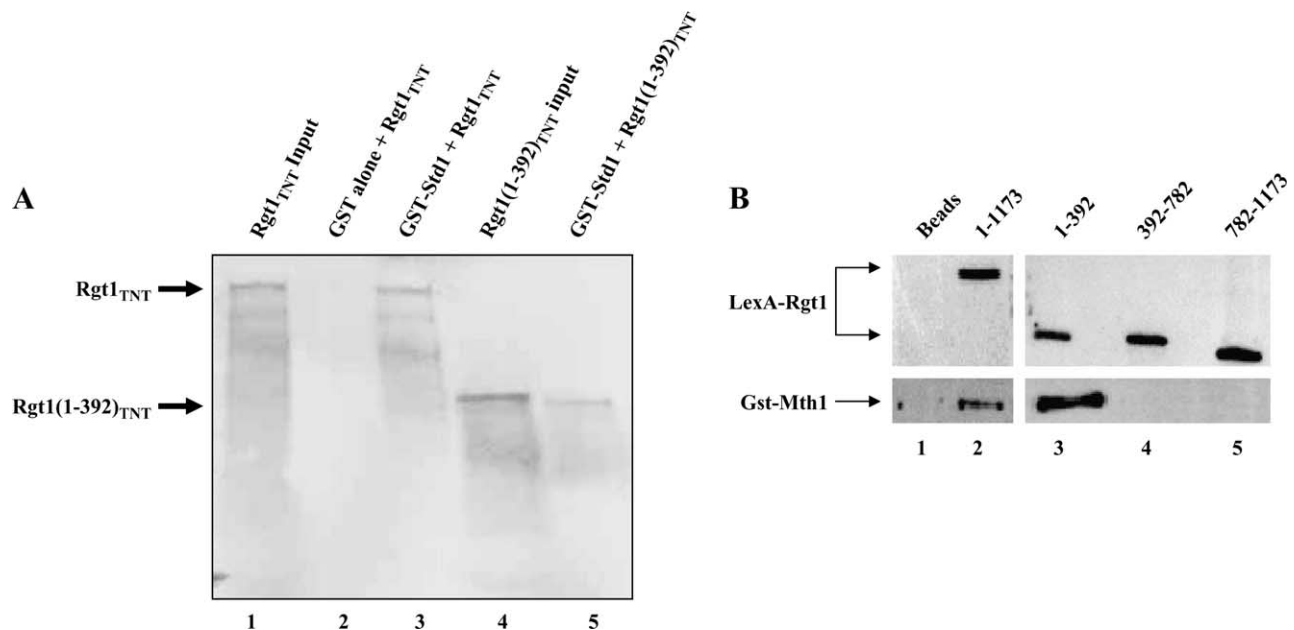


FIGURE 5.—Interaction between Rgt1 and Std1, Mth1. Agarose beads conjugated to glutathione were incubated with (A) GST-Std1 (produced in *E. coli* from pBM 3923) and (B) GST-Mth1 (pBM 4344) and processed as described in the legend to Figure 4. (A) Lane 1, 20% of the amount of Rgt1_{TNT} loaded over fusion proteins in lanes 2, 3, and 4; lane 2, GST alone (pBM 4266) incubated with Rgt1_{TNT}; lane 3, GST-Std1 incubated with Rgt1_{TNT}; lane 4, 20% of the amount of Rgt1(1-392)_{TNT} loaded over fusion proteins in lane 5; lane 5, GST-Std1 incubated with Rgt1(1-392)_{TNT}. (B) Different regions of LexA-Rgt1 [1–392 (pBM 3832), 392–782 (pBM 3833), and 782–1173 (pBM 3934)] were tested for their ability to bind to Mth1. The LexA beads and LexA-Rgt1 derivatives were incubated with GST-Mth1 and eluted as described in MATERIALS AND METHODS. The LexA-Rgt1 and GST-Mth1 were resolved by SDS-PAGE and visualized by Western blotting by using anti-LexA and anti-GST antibodies, respectively.

		2% Gal	Fold Repression	2% Glu	Fold Activation
Wild type	1 Empty vector	119.7 ± 20.6	-	236.1 ± 30.4	-
	2	15.6 ± 4.2	7.7	849.0 ± 76.7	3.6
	3	25.6 ± 4.8	4.7	35.9 ± 4.1	0.15
	4	20.4 ± 3.3	5.9	34.9 ± 4.9	0.15
	5	32.4 ± 5.0	3.7	32.1 ± 2.1	0.14
	6	28.5 ± 3.4	4.2	33.2 ± 4.6	0.14
	7	12.3 ± 3.5	9.7	14.5 ± 2.7	0.06
mth1Δ	8 Empty vector	145.8 ± 20.5	-	267.2 ± 24.0	-
	9	136.2 ± 19.7	1.1	904.9 ± 98.4	3.4
	10	13.2 ± 1.7	11.0	14.9 ± 3.4	0.06
	11	10.3 ± 2.3	14.2	15.2 ± 4.7	0.06

FIGURE 6.—Transcriptional repression/activation by various forms of Rgt1. Yeast cultures were grown in media as described in MATERIALS AND METHODS and assayed for β -galactosidase activity. The reporter of Rgt1 function used in this experiment is *HXT1-lacZ* (pBM 6243). Specific activity is calculated for both 2% galactose and 2% glucose conditions. The fold repression on 2% galactose is the β -galactosidase level of each strain (lines 2–7 and 9–11) divided into the β -galactosidase level of a strain with “empty vector” (lines 1 and 8, respectively). The fold activation on 2% glucose is the β -galactosidase level of each strain (lines 2–7 and 9–11) divided by the β -galactosidase level of a strain without Rgt1 (lines 1 and 8, respectively, empty vector). *RGT1* is fused to the *lexA* DNA-binding domain in all cases.

and 5B, lane 3, respectively). Thus, Std1 and Mth1 bind to the first 392 amino acids of Rgt1. These results, taken together, suggest that Rgt1 recruits Ssn6-Tup1, Mth1, and Std1 in the absence of glucose (on 2% galactose) to form a multiprotein repression complex.

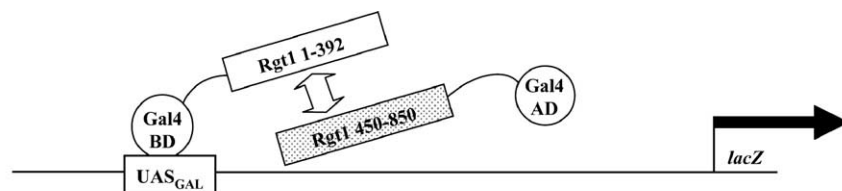
Roles of Mth1 and the central region of Rgt1 in regulating Rgt1 function: Phosphorylation of Rgt1 seems to inhibit its ability to repress transcription, because Rgt1



FIGURE 7.—Rgt1 is constitutively hypophosphorylated when Ser 758 is changed to Ala: yeast cells (YM 4509) expressing LexA-Rgt1 (pBM 3580) and LexA-Rgt1(S758A) (pBM 4446) were grown on minimal media containing either 2% galactose or 4% glucose. LexA-Rgt1 was immunoprecipitated and subjected to Western blot analysis using anti-LexA antibody as a probe.

becomes hyperphosphorylated upon addition of glucose to yeast cells (FLICK *et al.* 2003; KIM *et al.* 2003; LAKSHMANAN *et al.* 2003). We surmised that some of the *rgt1* deletion mutations that cause a constitutive repression phenotype (Table 2) may do so by preventing phosphorylation of Rgt1, so we searched those regions of Rgt1 for possible phosphorylation sites. Two of the several serine residues that we changed to alanine (S88 and S758) result in the same constitutive repression phenotype that is caused by deletion of these regions (*i.e.*, repression in cells growing on glucose, as well as on galactose; Figure 6, compare line 3 to line 4 and line 5 to line 6). Changing the serine at residue 758 to an alanine impairs its ability to be hyperphosphorylated in the presence of glucose (Figure 7). These results raise the possibility that phosphorylation of these residues is responsible for glucose-induced inhibition of repression by Rgt1.

Mth1 is required for repression by Rgt1 (OZCAN *et al.* 1993; SCHMIDT *et al.* 1999; SCHULTE *et al.* 2000; Figure 6, compare lines 2 and 9). However, Mth1 is not required for repression if the central regulatory domain



line	Gal4BD-Rgt1(1-392)	Gal4AD-Rgt1(450-850)	host genotype	β-galactosidase levels in strains grown on:	
				2% Gal	2% Glu
1	wt	AD only	wt	5.3 ± 0.8	3.8 ± 0.9
2	wt	wt	wt	5.4 ± 1.0	65.3 ± 10.3
3	wt	wt	<i>mth1Δ</i>	43.4 ± 5.8	54.6 ± 4.3
4	wt	wt	<i>MTH1-23</i>	3.1 ± 0.4	2.7 ± 0.9
5	wt	wt	<i>std1Δ</i>	3.4 ± 0.3	61.4 ± 6.8
6	wt	S758A(R ^c)	wt	5.0 ± 0.6	6.9 ± 1.2
7	S88A(R ^c)	wt	wt	4.3 ± 0.3	3.4 ± 0.8
8	wt	Δ510-520 (wt)	wt	4.2 ± 0.4	46.9 ± 8.7
9	wt	Δ520-530 (R ^c)	wt	6.2 ± 0.7	5.5 ± 4.3
10	wt	Δ530-540 (R ^c)	wt	4.5 ± 0.6	6.1 ± 7.6
11	wt	Δ630-640 (wt)	wt	1.3 ± 0.3	49.0 ± 6.9
12	wt	Δ640-650 (R ^c)	wt	7.2 ± 1.2	5.4 ± 0.3
13	wt	Δ650-660 (R ^c)	wt	5.0 ± 0.7	5.1 ± 1.6
14	wt	Δ810-820 (R ^c)	wt	3.4 ± 0.4	4.1 ± 0.8
15	wt	Δ820-830 (R ^c)	wt	4.3 ± 0.6	4.1 ± 0.4
16	wt	Δ830-840 (wt)	wt	2.9 ± 0.8	54.0 ± 6.9

FIGURE 8.—Glucose-dependent intramolecular interaction of Rgt1. Yeast cultures were grown and assayed for β-galactosidase activity as described in MATERIALS AND METHODS. The reporter of Rgt1 function used in this experiment is *Gal7-lacZ* (integrated into the genome at *MET2*, FM 353). The mutation present in the Gal4BD-Rgt1(1-392) and Gal4AD-Rgt1(450-850) plasmids, if any, is indicated, along with the phenotype it causes (R^c, constitutive repressor).

of Rgt1 is inactivated, either by deletion (Δ393–1171; Figure 6, line 11) or by changing the critical serine 758 to alanine (line 10). This suggests that Mth1 is not directly involved in transcriptional repression, but rather prevents the central regulatory region from interfering with transcriptional repression when glucose is absent.

Since we knew that relief of repression by Rgt1 is accompanied by its release from DNA (FLICK *et al.* 2003; KIM *et al.* 2003; LAKSHMANAN *et al.* 2003), we considered the idea that the central regulatory region of Rgt1 (520–830) inhibits function of the zinc cluster DNA-binding domain of Rgt1 (46–76) by direct contact. Indeed, we found that Rgt1 1–392 (fused to Gal4_{DBD}) interacts with Rgt1 450–850 (fused to Gal4_{AD}; Figure 8, line 2). This interaction is regulated by glucose, because it occurs only in glucose-grown cells. Mth1 regulates this intramolecular interaction of Rgt1: in cells lacking Mth1 the interaction occurs even in galactose-grown cells (line 3); in glucose-grown cells that contain Mth1 [due to the *MTH1-23* mutation, which renders Mth1 resistant to glucose-induced degradation (V. BRACHET and J. KIM,

unpublished observations)], the interaction is prevented (line 4). The central regulatory region of Rgt1 is required for the interaction, because it is abolished by the S758A mutation (line 6) and by several deletions of this region (lines 9, 10, 12–15). It is significant to note that all the mutations in this region that abolish the Rgt1 intramolecular interaction also lead to the constitutive repression phenotype (R^c), while the deletions that do not affect the interaction have no effect on Rgt1 function (lines 8, 11, and 16). This is consistent with our proposal (discussed below) that the central regulatory region of Rgt1 interferes with repression by interacting with and inhibiting its DNA-binding domain.

DISCUSSION

We identified domains of Rgt1 responsible for four functions.

1. A domain responsible for transcriptional repression resides between amino acids 210 and 250. Since these sequences are necessary for interaction of Ssn6 with

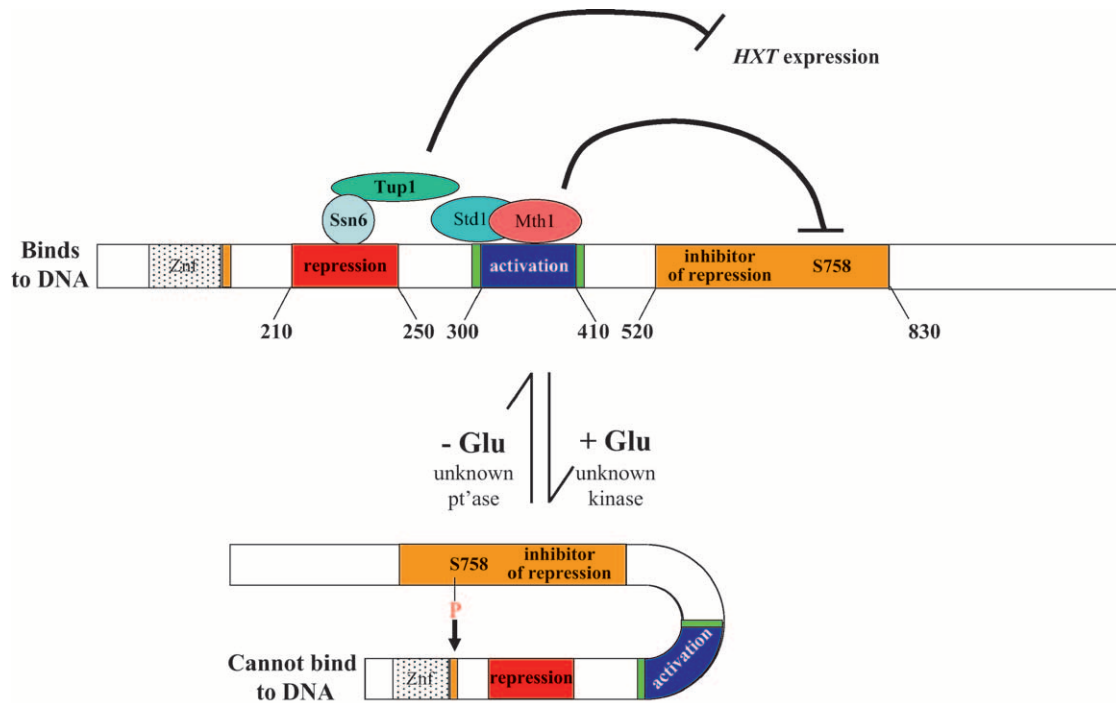


FIGURE 9.—Model for glucose regulation of Rgt1-mediated transcriptional repression. In the absence of glucose (bottom), Mth1 binds to Rgt1 and inhibits its phosphorylation, possibly at S88 and S758, and Rgt1 is able to bind to DNA. Addition of glucose induces degradation of Mth1, allowing phosphorylation of Rgt1 by a yet unidentified protein kinase. This results in an intramolecular interaction between the central regulatory region of Rgt1 and its N-terminal DNA-binding domain (which requires amino acids 80–90), preventing Rgt1 from binding to DNA and leading to derepression of gene expression.

Rgt1 (Table 3), it seems likely that this region of Rgt1 mediates repression by recruiting the Ssn6/Tup1 corepressor.

- Residues 320–390 contribute to transcriptional activation by Rgt1. None of these deletion mutations abolish transcriptional activation, suggesting that multiple regions of Rgt1 contribute to this function. We were unable to produce an Rgt1 missing all of its transcriptional activation domains, because most of the many large deletion mutations of *RGT1* that we constructed were undetectable in yeast cell extracts (presumably because they are unstable). Thus, definition of the Rgt1 transcriptional activation domain(s) requires further analysis.

Two central regions of Rgt1 serve to regulate its:

- Transcriptional activation.
- Repression.

Two 10-amino-acid segments (310–320 and 400–410) seem to inhibit transcriptional activation in galactose-grown cells, because deletions of these regions turn Rgt1 into a constitutive transcriptional activator (*i.e.*, even in cells grown on galactose; Table 2). Interestingly, residues 310–320 are also required for interaction of Std1 with Rgt1, suggesting that the role of Std1 may be to inhibit transcriptional activation, possibly by covering up this domain (see below).

The role of Std1 in regulating Rgt1 function remains unclear. Std1 is a paralog of Mth1 and, indeed, it is

partially functionally redundant with Mth1, because both proteins must be absent for Rgt1 to fully activate transcription in high glucose (SCHMIDT *et al.* 1999). However, Std1 and Mth1 have only partially overlapping roles in regulating Rgt1 function: Mth1, but not Std1, is required for DNA binding by Rgt1 and inhibits phosphorylation of Rgt1 (KIM *et al.* 2003). Std1 seems to negatively regulate Rgt1-mediated transcriptional activation [like Gal80 masks Gal4 activation (LUE *et al.* 1987; MA and PTASHNE 1987a; CHASMAN and KORNBERG 1990)].

The inhibition of the DNA-binding activity of Rgt1 by glucose presents a paradox: How can Rgt1 activate transcription of *HXT* genes when it is not bound to DNA? One possibility is that Rgt1 indirectly activates expression of *HXT* genes by binding to the promoter(s) of a gene(s) that encodes a protein necessary for their expression (MOSLEY *et al.* 2003). Alternatively, Rgt1 might bind to DNA in glucose-grown cells, but with an affinity too low for detection by the chromatin immunoprecipitation assay. It is also possible that transcriptional activation by Rgt1 is an artifact of the LexA-Rgt1 fusion protein with which this function was discovered (OZCAN *et al.* 1996). We do not favor this last idea because *rgt1* mutants have reduced expression of *HXT1* (OZCAN and JOHNSTON 1995), suggesting that it plays a role in activating expression of this gene. Clearly, this paradox remains to be resolved.

A large central portion of Rgt1 (520–830) and a short

segment adjacent to the zinc finger (80–90) operate to inhibit its transcriptional repression function in glucose-grown cells, because deletions of these regions cause Rgt1 to repress transcription even in glucose-grown cells (*i.e.*, they turn Rgt1 into a constitutive repressor; Table 2). It is significant that the deletions of the central regulatory region that abolish the intramolecular interaction lead to the constitutive repression phenotype, while deletion of adjacent residues that do not affect the interaction have no effect on regulation of repression by Rgt1 (Table 2; Figure 8). These results support the idea that the intramolecular interaction is responsible for relieving repression by Rgt1 in response to glucose.

Our observation that this central portion of Rgt1 (450–850) interacts with the N-terminal third of the protein (1–392) suggests that it directly inhibits transcriptional repression. Since glucose-induced relief of Rgt1-mediated repression is accompanied by release of Rgt1 from DNA (FLICK *et al.* 2003; KIM *et al.* 2003), perhaps the central regulatory region masks the zinc cluster DNA-binding domain of Rgt1, which lies near the N terminus (46–76). Indeed, sequences immediately adjacent to the zinc cluster (80–90) are required for this intramolecular interaction (data for S88A shown in Figure 8; data for Δ 80–90 not shown). The importance of this region to Rgt1 function is underscored by its extraordinary level of evolutionary conservation: amino acids 80–90 are nearly identical in the Rgt1 orthologs of yeast species as evolutionarily distant as *Kluyveromyces lactis*.

The intramolecular interaction does not occur in galactose-grown cells because Mth1 inhibits the interaction (Figure 8, line 3). The intramolecular interaction occurs in the presence of glucose probably because glucose induces degradation of Mth1 (FLICK *et al.* 2003; MORIYA and JOHNSTON 2004). Indeed, the interaction is not observed in a mutant (*MTH1-23*) in which glucose does not induce Mth1 degradation. Thus, removal of Mth1 from cells (either by addition of glucose to wild-type cells or by deletion of *MTH1*) causes the central regulatory region of Rgt1 to interact with its N-terminal portion.

Addition of glucose to cells results in hyperphosphorylation of Rgt1 (FLICK *et al.* 2003; KIM *et al.* 2003; MOSLEY *et al.* 2003) and derepression of Rgt1-repressed genes. In cells lacking Mth1, Rgt1 is hyperphosphorylated even in the absence of glucose (FLICK *et al.* 2003; LAKSHMANAN *et al.* 2003), suggesting that the role of Mth1 is to inhibit Rgt1 phosphorylation. It is notable that serines 88 and 758 are required for the intramolecular interaction, raising the possibility that phosphorylation of these residues is responsible for the interaction.

Our results, taken together, lead us to propose the model for regulation of Rgt1 function illustrated in Figure 9. In the absence of glucose, Mth1 is present in cells and binds to Rgt1 (possibly by contacting amino acids 350–360; Table 4, line 7) and inhibits its phosphorylation (possibly of S758 and/or S88). This prevents the

central regulatory region from interacting with the N-terminal portion of Rgt1, allowing Rgt1 to bind to DNA with its zinc cluster DNA-binding domain and repress transcription by recruiting the Ssn6/Tup1 corepressor complex to the repression domain (210–250). Addition of glucose to cells results in degradation of Mth1, which leads to phosphorylation of Rgt1. This stimulates the interaction of the central regulatory region with sequences near the DNA-binding domain, masking the DNA-binding domain and leading to derepression of *HXT* gene expression.

Rgt1 regulation combines mechanisms previously learned from other well-known transcription factors. It is phosphorylated in a regulated manner like Gal4 (MYLIN *et al.* 1989), undergoes a regulated conformational change like Leu3 (WANG *et al.* 1997, 1999), loses its affinity to bind DNA upon hyperphosphorylation like Crt1 (HUANG *et al.* 1998), and possibly uses Std1 to obscure an activation domain, like Gal4 uses Gal80 to mask its transcriptional activation domain (LUE *et al.* 1987; MA and PTASHNE 1987a; CHASMAN and KORNBERG 1990). These several layers of regulation bestow upon Rgt1 its complex regulation.

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