Rap1p Requires Gcr1p and Gcr2p Homodimers to Activate Ribosomal Protein and Glycolytic Genes, Respectively

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ABSTRACT

Efficient transcription of ribosomal protein (RP) and glycolytic genes requires the Rap1p/Gcr1p regulatory complex. A third factor, Gcr2p, is required for only the glycolytic (specialized) mode of transcriptional activation. It is recruited to the complex by Gcr1p and likely mediates a change in the phosphorylation state and/or conformation of the latter. We show here that leucine zipper motifs in Gcr1p and Gcr2p (1LZ and 2LZ) are each specific to one of the two activation mechanisms—mutations in 1LZ and 2LZ impair transcription of RP and glycolytic genes, respectively. Although neither class of mutations causes more than a mild growth defect, simultaneous impairment of 1LZ and 2LZ results in a severe synthetic defect and a reduction in the expression of both sets of genes. Intracistronic complementation by point mutations in the charged e and g positions confirmed that Gcr1p/Gcr1p and Gcr2p/Gcr2p homodimers are the forms required for the different roles of the activator complex. Direct heterodimerization between 1LZ and 2LZ apparently does not occur. Dichotomous Rap1p activation and its striking requirement for distinct homodimeric subunits give cells the capacity to switch between coordinated and uncoupled RP and glycolytic gene regulation.

Growth control in Saccharomyces cerevisiae appears to be mediated by the transcriptional activation function of Rap1p/Gcr1p, which regulates expression of glycolytic and translational component genes through the upstream Rap1p binding site (UAS_{RPG}) in each (Tornow et al. 1993; Shore 1994). The auxiliary factor Gcr2p (Uemura and Fraenkel 1990), which apparently does not contact DNA directly, is essential to the specialized mode of activation that also requires the Gcr1p binding site in DNA (the CT box; Zeng et al. 1997). This Gcr2p/CT box-dependent transcription is observed for most glycolytic promoters. In contrast, ribosomal protein genes and other translational component genes do not have CT boxes (Huie et al. 1992) and do not require Gcr2p. In these genes Gcr1p recruitment to the Rap1p DNA binding site is sufficient for activation (Santangelo and Tornow 1990; Tornow et al. 1993; Zeng et al. 1997). It therefore seems likely that the mild growth defect of \Delta gcr2 cells is caused by impairment of the specialized component of Gcr1p function (Zeng et al. 1997). In \Delta gcr1 cells, most if not all Rap1p activation is lost, and a severe growth defect results. Importantly, Gcr2p is inert in the absence of Gcr1p; its removal from \Delta gcr1 cells causes little or no worsening of the defects in cellular growth or activation of target genes.

We previously eliminated several models for GCR2 function in glycolytic gene expression. Gcr2p does not improve Rap1p/Gcr1p complex formation, strengthen Gcr1p binding to the CT box, or help to maintain wild-type steady-state levels of Gcr1p or Rap1p (Zeng et al. 1997). Like Gcr1p (Tornow et al. 1993), Gcr2p contains an activation domain (Uemura and Jigami 1992; our unpublished data). However, despite ample evidence that it physically associates with Gcr1p in vivo (see below), Gcr2p fails to augment either Gcr1p activation at UAS_{RPG} elements in the absence of a CT box or Gcr1lexAp activation at a lexA operator (Zeng et al. 1997). Since the current paradigm predicts that activation should be synergistic or at least additive under these circumstances (Ptashne and Gann 1997), this argues against the proposal (Uemura and Jigami 1992) that Gcr2p generally provides Gcr1p with an activation domain. At either Gcr1lexAp-bound lexA operators or at Rap1p/Gcr1p-bound UAS_{RPG} sites in the absence of a CT box, perhaps Gcr2p is somehow made activation incompetent (for example, see Halbach et al. 2000), or its recruitment by Gcr1p is blocked. We therefore cannot rule out the possibility that the Gcr2p activation domain might be dispensable even in specialized Gcr1p function at glycolytic genes.

Several lines of evidence suggest a new though poorly defined mechanism for GCR2 function. After Gcr2p is recruited by Rap1p/Gcr1p to the UAS_{RPG}/CT box sites in glycolytic genes, it may make an essential structural change in the complex (Zeng et al. 1997). First, two-hybrid analysis (Uemura and Jigami 1992)
and coimmunoprecipitation that we report here indicate that Gcr1p and Gcr2p bind to each other. Second, the requirements for the CT box (Baker 1991), for the CT-binding domain of Gcr1p (Hue et al. 1992), and for Gcr2p are equivalent. Removal of any or all of these components eliminates CT-dependent transcription (the CT box effect) without affecting Gcr1p function at GCR2, CT-independent promoters (Tornow et al. 1993; Zeng et al. 1997). Third, the loss of the CT box effect, and the accompanying mild growth defect of Δgcr2 cells, can be suppressed by numerous lesions in GCR1 (Uemura and Jigami 1995; Zeng et al. 1997). Many of these mutations map to the central serine/proline (SP)-rich region of Gcr1p, which appears to be heavily phosphorylated (Zeng et al. 1997; our unpublished data). Fourth, phosphorylation of Gcr1p, in particular the occurrence of a hyperphosphorylated form of the protein, is stimulated by Gcr2p (Zeng et al. 1997). The precise nature of the critical Rap1p/Gcr1p alteration(s) at CT boxes remains to be discovered, as does an explanation for the high frequency with which mutations in GCR1 cause Δgcr2 bypass. A thorough understanding of this unusual transcriptional activation complex, including its dichotomous behavior at different groups of target genes, may therefore hinge upon an elucidation of the molecular interactions between subunits.

We have found that Gcr2p, like Gcr1p (Deminoff et al. 1995), contains an excellent match to leucine zipper (LZ) motifs. We had expected 1LZ to function primarily in Gcr2p recruitment, due to the discovery of 2LZ and the fact that in-frame deletion of the Gcr1p leucine zipper (the GCR1Δ10 allele; Figure 1) causes a Δgcr2-like (mild) growth defect (Deminoff et al. 1995). A direct prediction of this idea was that combination of the GCR1Δ10 and Δgcr2 mutations should result in a defect no worse than that caused by either mutation alone. Interestingly, simultaneous deletion of 1LZ and Gcr2p instead leads to a severe growth defect similar to that of Δgcr1 cells. In the synthetically defective GCR1Δ10Δgcr2 strain, Rap1p/Gcr1p activation of both translational component genes (due to removal of 1LZ) and glycolytic genes (due to GCR2 deletion) is eliminated. This synthetic phenotype can be recapitulated by threonine replacements confined to the respective hydrophobic surfaces of 1LZ and 2LZ, confirming that simultaneous interference with Gcr1p/Gcr1p and Gcr2p/Gcr2p homodimer formation suffices to eliminate Rap1p/Gcr1p activation of most if not all target genes.

MATERIALS AND METHODS

Yeast methods: The following strains were used in this study: SD5, MATα leu2-3,112 his6 trp1-901 his3-200 TRP1:LEU2 Δgcr2::ura3 gcr1Δ4::URA3 SD6, same as SD5 but with GCR1Δ10 at the GCR1 locus and Ura3−; XZ12, MATα leu2-3,112 ura3-52 leu2::pH-L199(LEU2) Δtrp1::HIS3 Δgcr2::URA3. In SD5, GCR1 was replaced with URA3 in a spontaneous Ura3− version of DFY643 (Uemura and Fraenkel 1990). The GCR1Δ10 allele integrated into SD6 lacks codons 223–308 (Deminoff et al. 1995). Yeast cells were grown in liquid or on solid (2% agar) synthetic complete drop-out (SC) media or in rich (YEP) media, supplemented with 2% glucose (D) unless otherwise noted. The Yeastmaker kit from CLONTECH (Palo Alto, CA) was used for yeast transformation. When transformants were grown in SCD, selection for the plasmid was accomplished by dropping out the appropriate nutrient.

Plasmid construction: One multicopy [YEpTRP, TRP1 2 μ or Amp′ pMB1 ori (Tornow et al. 1993)] and two low-copy vectors [YCpTXZ, TRPI CEN ARS ori Amp′ pMB1 ori (Zeng et al. 1997); and Ycp50, URA3 CEN ARS ori Amp′ Tet′ pMB1 ori (Rose et al. 1987)] were used in this study. YCpTXZ/GCR2 was constructed by ligating a fragment of GCR2, extending from 300 bp upstream of the translational start codon to the genomic EcoRI site downstream of the stop codon, into the plasmid. Mutated variants of GCR2 were also carried on YcpTXZ. GCR2KΔ2 encodes the translational start codon in its proper location relative to upstream signals, followed immediately by codon 225 and the remainder of GCR2. The gcr2Δ2 allele replaces codons 223–481 with a linker sequence (GGCGCCGCC); gcr2Δ12 was a spontaneous isolate with a frameshift deletion of a single (CG) base pair in codon 509. Beginning at residue 509, the predicted (out-of-frame) amino acid sequence of gcr2Δ12 is lys-arg-arg-ser-gly-stop. In the four point-mutated versions of GCR2 (L2T, E2R, R2E, and E2R/R2E), codon 507 is changed from CAG to AGC, creating a Q to S amino acid change that does not affect Gcr2p function (not shown). YCpTXZ-(gcr2Δ2 + gcr2Δ12) carries both alleles in tandem. Care was taken to ensure that the results obtained with this plasmid, as well as those obtained with the combination of plasmids bearing GCR1Δ25 and GCR1Δ25 (see below), did not result from recombination between the two alleles (see results). The 2X hemagglutinin (HA) tag, inserted between GCR2 codons 356 and 357, and all point mutations described herein, were introduced using mutagenic oligonucleotides and either the polymerase chain reaction or the pAlter kit (Promega, Madison, WI). In each case, the resulting mutation was confirmed by DNA sequence analysis. Standard methods were used to introduce HA-tagged GCR2 downstream of the GCR1 gene in YEpTRP-GCR1-, -mycGCR1 (described previously in Tornow et al. 1993), and -mycGCR1Δ10 (see below); a version of GCR2 encoding an N-terminal fusion to the Escherichia coli lexA DNA-binding domain was similarly introduced. GCR1Δ10 has been described previously (Deminoff et al. 1995). GCR1Δ25, GCR1Δ25 and GCR1Δ27 were carried on YcpxTZ, while GCR1Δ25 was carried on Ycp50, each as a fragment comprising sequences between the genomic SacI and XhoI sites, as is wild-type GCR1 on all plasmids used in this study. GCR1Δ25 and GCR1Δ25 contain a 2X myc tag and behave indistinguishably from untagged versions (our unpublished data). A set of four plasmids was constructed by ligating the SacI-XhoI fragment of GCR1 or (GCR1Δ27) into the XhoI site of YcpTXZ-GCR2 (or -GCR2Δ27); YcpTXZ-(GCR1Δ25 + GCR1Δ27) was constructed in an analogous way. A myc-tagged version of GCR1Δ27 was ligated into YEpTRP in the same orientation as wild-type GCR1. The point mutations in GCR1Δ27 were also recombined with the fusion gene GCR1lexA [also carried on YEpTRP; see Tornow et al. 1993] for a detailed description of myc- and lexA-tagged GCR1 alleles.

Primer extension, immunoprecipitation, Western blotting, and band retardation: Oligonucleotides specific for glycolytic genes, ribosomal protein genes, or ACT1 were labeled, primer extension reactions were done, and products were analyzed by using standard methods (Triezenberg 1992). Immunoprecipitation with α-myc antibody was done as described pre-
RESULTS

2LZ is required for Gcr2p function: We previously reported that Gcr1p contains an excellent match to LZ motifs and demonstrated that it is both necessary and sufficient for Gcr1p homodimerization (Deminfoff et al. 1995). Since leucine zippers are also known to mediate heteromultimer formation (Landschulz et al. 1988; Gentz et al. 1989; O'Shea et al. 1989), and Gcr2p is recruited by Gcr1p to target promoters, we looked for the same molecule (Gcr2pE2#R2#), which should destabilize a Gcr1p/Gcr2p heterodimer but not a Gcr2p/Gcr2p homodimer (compare Figure 3B with 3A), function is restored (Figure 4).

Reciprocal charge switches in 1LZ and 2LZ complement intracistronically and by second site reversion: The L2T, E2R, or R2E mutations in GCR2 each lead to a growth defect equivalent to that of Δgcr2 strains (Figure 4); the steady-state levels of these mutated GCR2 products are indistinguishable from that of wild-type Gcr2p (not shown). This confirms that the predicted 2LZ structure is required for Gcr2p function. Surprisingly, when charges are reversed in both the e and g positions in the same molecule (Gcr2pE2#R2#), which should destabilize a Gcr1p/Gcr2p heterodimer but not a Gcr2p/Gcr2p homodimer (compare Figure 3B with 3A), function is restored (Figure 4).

The gcr2#E2, gcr2#R2, and GCR2#E2#R2 mutations should

Figure 1.—Diagram of Gcr1p and Gcr2p. The N terminus (open box), central Gcr1p-homologous region (2H; shaded box), and C-terminal leucine zipper (2LZ, checkered box) are indicated within the predicted primary sequence of Gcr2p (a total of 534 residues). In Gcr1p (785 residues), hypomutatable regions A, B1, B3, and C (solid boxes) are indicated; region D (checkered box) coincides with 1LZ (Deminfoff et al. 1995). The central serine/proline (SP)-rich region (shaded box), which shares homology with the 2H region of Gcr2p, and the CT box-binding domain (open box) are also shown.
all destabilize a heterodimeric interaction with wild-type Gcr1p by setting up charge repulsions (Figure 3B). Since \(GCR2^{L2T}\) is a functional allele, the charged residues in positions \(e\) and \(g\) appear to participate in Gcr2p homodimerization (Figure 3A) rather than Gcr1p/Gcr2p heterodimerization (Figure 3B). We tested this idea by taking advantage of the inability of either the \(gcr2^{E2R}\) or the \(gcr2^{R2E}\) product to complement the \(\Delta gcr2\) defect. A low-copy plasmid was constructed that contained both \(gcr2^{E2R}\) and \(gcr2^{R2E}\) (in tandem; see MATERIALS AND METHODS). Transformation with this plasmid restored wild-type growth to \(\Delta gcr2\) cells by intracistronic
complementation (Figure 4). Reisolation of the complementing plasmid from yeast cells, followed by DNA sequence analysis of the rescued alleles, demonstrated that the Gcr2<sup>+</sup> phenotype did not result from recombination between gcr2<sup>E2R</sup> and gcr2<sup>E2E</sup> (not shown). Intracistronic complementation between these two GCR2 alleles rules out the idea that coiled-coil formation between 1LZ and 2LZ makes a significant contribution to Gcr1p/Gcr2p function and suggests instead that position e and g charged residues in 2LZ support Gcr2p homodimerization.

We next used an analogous genetic approach to test whether 1LZ participates in Gcr1p/Gcr2p heterodimerization or Gcr1p/Gcr1p homodimerization. A definitive answer was obtained in the SD6 background (see below). As expected, 1LZ could be inactivated by point mutations that disrupt potential salt bridges between residues in the e and g positions [E270K or E277K (E2K); Figures 3C and 5]. In complete agreement with the results of 2LZ analysis, intracistronic complementation was observed between GCR1E2K and GCR1K2E (Figure 5). This result independently rules out the possibility that 1:1 heteromeric leucine zipper formation between 1LZ and 2LZ plays a role in Gcr1p/Gcr2p function.

**Gcr1p/Gcr2p complex formation does not require 1LZ:** Since 2LZ function does not require coiled-coil formation with 1LZ (and vice versa), we tested whether 1LZ is needed at all for association between Gcr1p and Gcr2p. If not, it should be possible to detect Gcr1p/Gcr2p complexes in the absence of 1LZ. We had previously shown that deletion of 1LZ leads to loss of the slow but not the fast-migrating complex between the fusion protein Gcr1lexAp and the lexA operator in band retardation analysis (Deminoff et al. 1995). These and other data demonstrated that removal of 1LZ eliminates Gcr1p homodimerization. We found that the GCR1<sup>L27T</sup> product (L267T + L274T + L281T; boxed residues in

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**Figure 4.—Effect of substitutions for hydrophobic or charged residues in 2LZ.** XZ12 (GCR1Δgcr2) cells, transformed with a low-copy TRPI plasmid (YCpTXZ or YCpTXZ-GCR2 derivative), were streaked onto SCD plates and grown for 2 days at 30°C. Each plasmid contained the indicated allele(s), as follows: GCR2, wild type; gcr2<sup>E2T</sup>, point mutations I497T, L500T, and L504T; gcr2<sup>2E2E</sup>, point mutations E503R and E510R; gcr2<sup>E2E</sup>, point mutations R508E and R515E; GCR2<sup>E2R</sup> and E503R and E510R; GCR2<sup>E2R</sup> and E503R and E510R, R508E, and R515E; gcr2<sup>2E2E</sup>, two alleles in tandem, one with E503R and E510R, the other with R508E and R515E (see text); Vector, no GCR2 gene.
Figure 5.—Intracistronic complementation between GCR1 alleles encoding reciprocal mutations in charged amino acids in 1LZ. The numbered segments of this SCD plate contain colonies of SD6 cells transformed with low-copy plasmids, as follows: 1, YCp50-GCR1<sup>1</sup> YCpTXZ-GCR2<sup>1</sup>; 2, YCp50-GCR1<sup>1</sup> YCpTXZ; 3, YCp50 + YCpTXZ-GCR<sup>12K</sup><sup>ΔD</sup>; 4, YCp50-GCR1<sup>12K</sup><sup>ΔD</sup> + YCpTXZ; 5, Yc50 + YCpTXZ; or 6, YCp50-GCR1K2E<sup>1</sup> YCpTXZ. See the text and Figure 3B for the amino acid substitutions encoded by these alleles. The transformants were grown for 3 days at 30°.

Figure 6.—Point mutations in 1LZ impair Gcr1p homodimerization. The fusion protein Gcr1lexAp (Tornow et al. 1993), with an intact (wt, lanes 2–5), point-mutated (L2T, lanes 6–9), or deleted (ΔD, lanes 10–13) leucine zipper, was expressed from the multicopy plasmid YEpTRP. GCR1<sup>L2T</sup> encodes the substitutions L267T, L274T, and L281T in the hydrophobic surface of 1LZ (see Figure 3B). Band retardation assay (Figure 6, lanes 6–9) but forms complexes with Gcr2p just as effectively as wild-type Gcr1p according to the coimmunoprecipitation assay (Figure 7, compare lanes 1 and 2). Unless the use of overexpressed polypeptides results in artifactual complex formation, association between Gcr1p and Gcr2p does not require 1LZ. In contrast, we have not yet detected Gcr1p/Gcr2p complexes in the absence of 2LZ (see below).

Simultaneous impairment of 1LZ and 2LZ creates a synthetic growth defect: As part of the analysis of GCR2 function, we constructed a mutant strain in which Δ<sub>gcr2</sub> cells were transformed with a low-copy plasmid carrying GCR1<sup>ΔD</sup> (the 1LZ-deleted allele; Deminoff et al. 1995), expecting that combination of the Δ<sub>gcr2</sub> and GCR1<sup>ΔD</sup> mutations would result in a defect no worse than that caused by either mutation alone (see Introduction). We were intrigued to find that this GCR1<sup>ΔD</sup> Δ<sub>gcr2</sub> genotype (Figure 8A, segment 1), like the equivalent SD6 genotype (see MATERIALS AND METHODS), results in a synthetic growth defect nearly as severe as that of Gcr1<sup>2Δ</sup> strains (Figure 8A, segments 2 and 3; note that adding GCR2 to the Δ<sub>gcr1</sub> background does not improve growth). As expected, addition of both GCR1 and GCR2 to SD5 cells on the low-copy plasmid YCpTXZ restores wild-type growth (Figure 8A, segment 4), and addition of YCpTXZ-GCR1 or YCpTXZ-(GCR1<sup>ΔD</sup> + GCR2) partially improves the growth rate (Figure 8A, segments 5 and 6, respectively). Western analysis demonstrated that steady-state levels of the GCR1<sup>ΔD</sup> product are unaffected by removal of Gcr2p (not shown). Reduced Gcr1p levels therefore cannot explain the synthetic phenotype.

The effect of point mutations in 1LZ and 2LZ suggested that the synthetic defect might be explained by the combined loss of Gcr1p/Gcr1p and Gcr2p/Gcr2p homodimers. If so, it should be possible to reconstitute the defect with point mutations confined to the predicted hydrophobic surfaces of 1LZ and 2LZ. We therefore combined the GCR2 allele containing the L2T mutation in 2LZ with the analogous GCR1<sup>L2T</sup> allele. As expected, the latter inactivates 1LZ (Figure 6) and causes a mild growth defect (Figure 8B); i.e., it phenocopies GCR1<sup>ΔD</sup> (Deminoff et al. 1995). Combination of GCR1<sup>L2T</sup> and gcr2<sup>L2T</sup> did indeed recapitulate the synthetic defect (Figure 8B).

We next sought an explanation for the synthetic phenotype by quantitating transcription of Rap1p/Gcr1p target genes. In isolation, deletion of 1LZ (GCR1<sup>ΔD</sup>) resulted in decreased mRNA levels of the ribosomal protein genes RPS2, RPS14A, and RPL11B (Figure 9, compare lanes 1 and 3), and had little or no effect on glycolytic transcripts (compare lanes 6 and 8). As shown previously (Tornow et al. 1993; Zeng et al. 1997), the
Figure 7.—1LZ is not required for Gcr1p/Gcr2p coimmunoprecipitation. Epitope-tagged (myc or HA) or untagged (−) versions of Gcr1p and Gcr2p were carried in tandem on the multicopy plasmid YEpTRP. The myc-tagged variant of Gcr1p was wild type (WT, lanes 1 and 3) or contained substitutions (L267T, L274T, L281T) for hydrophobic amino acids in 1LZ (L2T, lane 2; see Figure 3B). Protein from cell extracts was immunoprecipitated with α-myc antibody. Eluted proteins were separated by SDS-PAGE, blotted to nitrocellulose, and subjected to Western analysis with α-myc (top) or α-HA (middle) antibody. Western analysis of HA-tagged Gcr2p from the crude extracts is shown at the bottom (input).

isolated Δgcr2 mutation had the opposite effect: there was little or no decrease in ribosomal protein gene transcription (Figure 9, compare lanes 1 and 2), while levels of the glycolytic transcripts ENO1, ENO2, and PYK1 were reduced (compare lanes 6 and 7). Combination of the GCR1ΔN and Δgcr2 mutations appears to result in a simple composite phenotype resembling that of Δgcr1 strains (Figure 9, compare lanes 4 and 5 and lanes 9 and 10). The decrease in steady-state levels of enolase, pyruvate kinase, and other glycolytic enzymes is equally severe in Δgcr2 and Δgcr1 backgrounds (Uemura and Fraenkel 1990; our unpublished data), confirming that this composite phenotype does not result from a synthetic loss of glycolytic enzyme activity.

Mutations in 2LZ eliminate Gcr1p/Gcr2p complex formation: We next took advantage of a set of constructs, originally designed to analyze Gcr2p activation, which encode the lexA DNA-binding domain fused to the N terminus of Gcr2p. We used this series of fusion constructs to test whether mutations in Gcr2p, specifically in 2LZ, eliminate coimmunoprecipitation with Gcr1p. Extracts from strains expressing myc-tagged or untagged Gcr1p and, on the same high-copy vector, variants of lexA-Gcr2p, all of which are expressed and stable (not shown), were immunoprecipitated with α-myc antibody. The products encoded by lexA-GCR2 wild type, -GCR2ΔN, and -gcr2Δ2H were all coimmunoprecipitated by the antibody in the presence of the myc tag on Gcr1p (Figure 10, lanes 1–3), but not in its absence (lane 8). However, one deletion and several inactivating point mutations in 2LZ (lanes 4–7) eliminated association with Gcr1p. This is consistent with the idea that Gcr1p/Gcr2p complex formation is required for Gcr2p function.

Figure 8.—Impairment of 1LZ and 2LZ causes a synthetic defect. (A) The double mutant SD5 (Δgcr1 Δgcr2) was transformed with YCpTXZ derivatives that conferred each of the indicated genotypes or with vector alone (see text for details). The equivalent mild growth defect of GCR1ΔN GCR2 and GCR1 Δgcr2 cells, relative to wild type (GCR1 GCR2), is apparent by streaking onto SC plates and incubating at 30°C for 2 days. (B) The growth defects described above are recapitulated at the level of point mutations in 1LZ and 2LZ. Strain SD5 (Δgcr1Δgcr2) was transformed with vector (YCpTXZ) alone or by carrying a combination of GCR1 and GCR2 alleles (either WT or with the GCR1ΔN mutations, gcr2Δ2H mutations, or both; see Figure 3, A and C). Cells were streaked onto SC plates and incubated at 30°C for 2 days.

DISCUSSION

We report here a molecular analysis of the leucine zipper motifs in Gcr1p (1LZ) and Gcr2p (2LZ). The L2T point mutations essentially substitute a hydroxyl for two methyl groups (Thr replacing Leu or Ile) at each of three positions in the hydrophobic interface of 1LZ or 2LZ (Figure 3). These L2T alleles cause a mild growth defect, comparable to that resulting from dele-
### Figure 9

Removal of 1LZ or Gcr2p leads to distinct gene expression defects. Low-copy plasmids were introduced into strain SD6 to generate the indicated genotype (WT, wild type; ΔD, deletion derivative lacking 1LZ; ΔD, null) and GCR2 (WT, wild type; ΔD, null). Ten micrograms of total RNA from each transformant was analyzed by primer extension. Lanes 1–5, primers capable of quantitating transcripts initiating in RPS2, RPS14A, RPL11B, or ACT1 (as a control) were used. Lanes 6–10, primers capable of quantitating transcripts initiating in ENO1, ENO2, PYK1, or ACT1 (as a control) were used.

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<th>Gene</th>
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<td>RPS2</td>
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<td>RPS14A</td>
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<td>RPL11B</td>
<td>1 2 3 4 5</td>
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<tr>
<td>ACT1</td>
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### Figure 10

Mutations in 2LZ eliminate Gcr1p/Gcr2p association. Extracts from SD5 cells, transformed with a multicopy vector that carries both GCR1 [myc-tagged (myc, lanes 1–7) or with no tag (NT, lane 8)] and variants of lexA-GCR2 (see text and Figure 3A for a description of each GCR2 deletion and point mutation shown), were immunoprecipitated with α-myc antibody. The immunoprecipitates were then analyzed by Western blotting using α-lexA antibody, the results of which are shown. Each lexA-GCR2 variant encodes a stable product, as determined by Western blotting of the crude extracts used for immunoprecipitation (not shown). The full-length version of the R2E variant was unstable; a stable ΔN derivative was used for this analysis. Its predicted position in lane 7 is indicated by an open arrow. The shaded arrow indicates the position of wild-type lexA-Gcr2p.

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<tr>
<td>Gcr1p</td>
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<td>LexA-Gcr2p</td>
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3B with any heterologous leucine zipper: the conclusions drawn from reciprocal second site reversion and/or intracistronic complementation are identical. Charge switching, preferably confirmed by biochemical analysis, should therefore be a generally applicable method to distinguish homodimerization from heterodimerization. A possible limitation is that three-stranded or four-stranded coiled coils might be difficult to resolve with this genetic test; tetrameric coiled coils have been crystallized and may have functional significance in other systems (HARBURY et al. 1993). Nevertheless, it is unlikely that 1LZ and 2LZ are unusual structures in their sensitivity to charge switch analysis. Since e and g positions in coiled coils are known to harbor both attractive and repulsive effects on dimerization (Hu et al. 1993), most leucine zipper complexes should be decipherable with this method. Charge switch analysis of leucine zippers should be possible even if disruption of the α-helix in question causes no obvious phenotype; fusion of an LZ to the basic segment of a bZIP motif and measurement of the DNA-binding capacity of the resulting chimera were used successfully in an analogous study of the LR domain in Vpr, a 15 kD late viral gene product of HIV-1 (WANG et al. 1996).

Despite the progress in understanding 1LZ and 2LZ function, the surfaces in each polypeptide that mediate Gcr1p/Gcr2p complex formation remain unidentified. In Gcr1p, the residues that specify interaction with Gcr2p appear to lie entirely outside of 1LZ (which coincides with hypomutable region D; Figure 1) or are located redundantly within 1LZ and elsewhere: communoprecipitation of Gcr2p by wild-type Gcr1p and the GCR1227p product are indistinguishable (Figure 7). Furthermore, the Gcr2p-dependent genes ENO1, ENO2, and PYK1 are affected little, if at all, in the GCR1Δb GCR2 background (Figure 9), which also suggests that Gcr2p recruitment occurs in the absence of 1LZ. It is tempting to speculate that one or more of the remaining GCR1 hypomutable regions (A, B1, B3, or C; Figure 1) harbor the Gcr2p contact domain. For example, point mutations in hypomutable region A eliminate Gcr2p-dependent protection of GCR1 products made unstable by the Q15R lesion (our unpublished data). However, the possibility of an essential interaction between Gcr2p and residues C-terminal to 1LZ cannot yet be eliminated.

We have not yet successfully communoprecipitated Gcr1p/Gcr2p complexes in the absence of 2LZ function (Figure 10). It is therefore possible that 2LZ is required for contact with Gcr1p; i.e., it contacts a domain in Gcr1p outside of 1LZ. The most likely way for this to occur would be through positions b, e, or f in 2LZ.
or some combination thereof; these residues should remain exposed after 2LZ homodimerization. Of course, the idea that interaction with Gcr1p explains the requirement for 2LZ homodimers in GCR1 function remains only one of several possibilities.

A recent microarray filter hybridization experiment (Lopez and Baker 2000) in which cells were grown on a nonfermentable carbon source (glycerol plus lactate) failed to identify several Gcr1p-dependent genes that we have consistently found are 2- to >20-fold less well transcribed in several different Δger1 backgrounds (RPS2, RPS14A, RPS3, RPL11B, RPL30, TEF1, TEF2, and ADH1; Santangelo and Tornow 1990; Tornow and Santangelo 1990; Tornow et al. 1993; Zeng et al. 1997; Figure 9 and our unpublished data). ADH1 is a particularly striking example—it is even more strongly Gcr1p dependent in cells grown on pyruvate (>20-fold) than in those grown on glucose (7- to 8-fold), yet it fell below the 2-fold benchmark chosen by Lopez and Baker for GCR1 dependence. It would appear that their filter hybridization assay is not well suited for quantitative analysis; otherwise it is difficult to account for the dramatic discrepancy between their results and previously published data obtained with more sensitive methodologies.

Perhaps a microchip analysis in which glucose, pyruvate, and/or other nonfermentable compounds are each tested as the carbon source would yield more accurate quantitation (Bartosiewicz et al. 2000) and a clearer picture of the genome-wide effect of GCR1 deletion.

Our data rule out the idea that the Δger1 effect on translational component gene expression is an indirect consequence of a slow growth rate (Huie et al. 1992), since GCR1Δ gcr2 strains are only mildly growth defective (Figure 8A; Deminoff et al. 1995) but exhibit a Δger1-like loss of RPS2, RPS3, RPS14A, RPL11B, and RPL30 and TEF1 and TEF2 transcription (Figure 9, compare lanes 3 and 5; our unpublished data). Moreover, reduced glycolytic enzyme levels are insufficient to explain the Δger1 growth phenotype, since similar reductions in Δger2 cells have a much milder effect on growth (Uemura and Franke1990). Therefore, although the central role of UASnon-bound Rap1p in glycolytic gene expression may indeed be to recruit Gcr1p/Gcr2p complexes to the adjacent CT box and permit a specialized mode of activation, it is equally true that Rap1p independently stimulates transcription of translational component genes by recruiting Gcr1p in the absence of a CT box. The disparity in the requirements for Gcr1p and Gcr2p domains in these two mechanisms of activation could not be more striking (Table 1). We therefore remain convinced (Santangelo and Tornow 1990; Tornow et al. 1993; Zeng et al. 1997) that Rap1p potentiates transcription at UASnon sites by doing more than just clearing chromatin so that other activator proteins can bind DNA (Yu and Morse 1999).

The mechanistic dichotomy of Rap1p/Gcr1p function at translational component and glycolytic genes suggests that independent control of these two classes of regulatory targets is a useful feature of growth control. Recent analysis of genome-wide expression patterns suggests that, under the conditions tested so far, most translational component and glycolytic genes are coregulated. For example, coordinated downshifts appear to occur during the diauxic transition, heat-shock response, and sporulation time courses (Derisi et al. 1997; Eisen et al. 1998). It remains to be seen if a regulatory scenario can be found in which the CT-dependent and CT-independent modes of Rap1p/Gcr1p activation respond differentially. Regardless, it is probably significant that the Gcr1– condition appears to be a unigenic synthetic phenotype: the combined Gcr1p-stimulated fractions of translational component and glycolytic genes have a synergistic effect on both the growth rate (Figure 8) and the protein synthesis rate (our unpublished data). This arrangement should allow fine-tuned growth regulation in response to nutrient availability and other stimuli over a wide range. We are therefore particularly interested in the possibility that phosphorylation of

![Figure 11](image-url)
Gcr1p or Gcr2p (or both) through the Ras signal transduction pathway is an important feature of Rap1p transcriptional activation (see Klein and Struhl, 1994; Zeng et al., 1997). Given its demonstrated effect on Cln expression (Tokiwa et al., 1994), the Ras pathway may therefore represent the predominant regulatory link between the growth and cell cycles.

These results extend our working model for Gcr2p function, that a Gcr2p-dependent alteration to Rap1p/Gcr1p complexes mediates Gcr1p activation in the presence of a combinatorial UAS_{src}/CT box element (Zeng et al., 1997). The data that we report here, especially the identification of a synthetic growth defect resulting from a combination of mutations that inactivate ILZ (loss of translational component gene expression) and 2LZ (loss of glycolytic expression), confirm the idea that Rap1p activates transcription at UAS_{src}, through two distinct mechanisms. An updated version of our working model is depicted in Figure 11. This model explains why the Δger1 and Δger2 mutations, which result in an equivalent decrease in glycolytic enzyme levels, cause growth defects that differ greatly in magnitude. In the absence of a CT box adjacent to UAS_{src}, ILZ (unlike the CT-binding domain or Gcr2p) is essential; in the presence of a CT box, those requirements are neatly reversed. A full understanding of these observations should elucidate other important features of this system, perhaps including the mystery surrounding the other duality in Rap1p function: its capacity to act as both an activator and a repressor of transcription.

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