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A Transcriptional Switch in the Expression of Yeast Tricarboxylic Acid Cycle Genes in Response to a Reduction or Loss of Respiratory Function†

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The Hap2,3,4,5p transcription complex is required for expression of many mitochondrial proteins that function in electron transport and the tricarboxylic acid (TCA) cycle. We show that as the cells' respiratory function is reduced or eliminated, the expression of four TCA cycle genes, *CIT1*, *ACO1*, *IDH1*, and *IDH2*, switches from *HAP* control to control by three genes, *RTG1*, *RTG2*, and *RTG3*. The expression of four additional TCA cycle genes downstream of *IDH1* and *IDH2* is independent of the *RTG* genes. We have previously shown that the *RTG* genes control the retrograde pathway, defined as a change in the expression of a subset of nuclear genes, e.g., the glyoxylate cycle *CIT2* gene, in response to changes in the functional state of mitochondria. We show that the *cis*-acting sequence controlling *RTG*-dependent expression of *CIT1* includes an R box element, GTCAC, located 70 bp upstream of the Hap2,3,4,5p binding site in the *CIT1* upstream activation sequence. The R box is a binding site for Rtg1p-Rtg3p, a heterodimeric, basic helix-loop-helix/leucine zipper transcription factor complex. We propose that in cells with compromised mitochondrial function, the *RTG* genes take control of the expression of genes leading to the synthesis of α-ketoglutarate to ensure that sufficient glutamate is available for biosynthetic processes and that increased flux of the glyoxylate cycle, via elevated *CIT2* expression, provides a supply of metabolites entering the TCA cycle sufficient to support anabolic pathways. Glutamate is a potent repressor of *RTG*-dependent expression of genes encoding both mitochondrial and nonmitochondrial proteins, suggesting that it is a specific feedback regulator of the RTG system.

Cells reconfigure their pattern of gene expression to accommodate changes in nutrient availability. Often, this is seen as an induction of genes that enable cells to utilize certain nutrients or as a repression of those genes when such nutrients are no longer available. Cells also reconfigure patterns of gene expression in response to different stress conditions, for instance, heat shock or osmotic stresses, as a mechanism of protection against those environmental insults. In cells of the budding yeast *Saccharomyces cerevisiae*, dramatic changes in gene expression are observed when cells switch from fermentative to oxidative metabolism, as in the diauxic shift, when cells growing on glucose—a repressing carbon source—begin to deplete the glucose from the medium (8). The expression of many nucleus-encoded mitochondrial proteins, such as components of the electron transport chain and enzymes of the tricarboxylic acid (TCA) cycle, become derepressed during the diauxic shift. The derepression of many of these proteins requires either Hap1p, an oxygen-sensing transcriptional activator (6, 29), the heteromeric Hap2,3,4,5p transcriptional complex (10, 11, 24, 26, 27, 30, 43), or both of these *trans*-acting regulators. Thus, the *HAP* system represents an important mechanism for global control of expression of key components of respiratory metabolism.

Yeast cells also modulate the expression of nuclear genes in response to mitochondrial dysfunctions (28). This interorganelle signaling pathway, called retrograde regulation, can be thought of as a stress response whose function is to accommodate various cellular activities to the changes in the mitochondrial state (39). The best-studied example of the retrograde response is that of the *CIT2* gene, whose expression is sensitive to conditions or mutations that compromise mitochondrial functions, such as inhibition of respiration, loss of TCA cycle activity, or loss of mitochondrial DNA (3, 18, 19). Recently, we found a new cytosolic D-lactate dehydrogenase activity encoded by a previously uncharacterized gene, *YEL071w*, now named *DLD3*, that shows a similar retrograde response as *CIT2*, namely, increased expression in cells with dysfunctional mitochondria (4). *CIT2* encodes a peroxisomal isoform of citrate synthase (CS2) that functions in the glyoxylate cycle; CS2 shares 75% sequence similarity with the mitochondrial citrate synthase (CS1) encoded by the *CIT1* gene, suggesting that *CIT1* and *CIT2* arose by a duplication of some ancestral citrate synthase gene. In cells with compromised mitochondrial functions, for example, those without mitochondrial DNA ([*rho*^{0}] petites), *CIT2* expression is elevated by as much as 30- to 40-fold (19). Physiological studies suggest that this increase in *CIT2* expression facilitates a more efficient utilization of carbon via the transfer of metabolites from the glyoxylate cycle to the TCA cycle (40). In contrast to *CIT2*, the level of *CIT1* expression is unaffected by the loss of mitochondrial DNA (18).

The expression of *CIT2* and *DLD3* is dependent under all conditions tested on three genes, *RTG1*, *RTG2*, and *RTG3*. *RTG1* and *RTG3* encode basic helix-loop-helix/leucine zipper (bHLH/Zip) transcription factors that bind as a heterodimer to activate transcription to a novel DNA target site, GTCAC, called an R box (14). *CIT2* and *DLD3* contain two R boxes in their 5′ noncoding regions, both of which are required for maximal gene expression. *RTG2* encodes a novel cytoplasmic protein that has an amino-terminal ATP binding domain sim-

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† This paper is dedicated to our friend and colleague, Paul Srere.

ilar to the hsp70/actin/sugar kinase superfamily of ATP binding proteins (2). Rtg2p also shares some sequence similarity with bacterial polyphosphatases and enzymes that hydrolyze guanosine tetra- and pentaphosphate (16). Although the precise function of Rtg2p is unknown, genetic data suggest that it acts upstream of Rtg1p and Rtg3p in the control of gene expression (36). The *CIT2-DLD3* retrograde response appears to be controlled by an Rtg2p-dependent redistribution of the Rtg1p-Rtg3p transcriptional complex from a predominantly cytoplasmic location in cells with robust mitochondrial function to a nuclear location in cells whose mitochondrial functions have been compromised, such as in [*rho*^{0}] petites (38).

None of the *RTG* genes are essential, nor are they required for growth of cells on some nonfermentable carbons sources. Although the *RTG* genes are required for both basal and retrograde expression of *CIT2* and *DLD3*, two unexpected phenotypes of the *rtg* mutants were observed: an inability of cells to grow on acetate, and a growth requirement for glutamate and aspartate on minimal glucose medium (18). These phenotypes are characteristic of cells with blocks in both the TCA and glyoxylate cycles. The inability to grow on acetate is a common phenotype of cells lacking TCA cycle enzymes (23), and *cit1 cit2* and *aco1* mutants are glutamate auxotrophs (12, 15). These observations suggest a potential defect in the TCA cycle in *rtg* mutant cells. Biochemical experiments have suggested that *rtg* mutant cells may have multiple and cumulative lesions in the TCA cycle that impair the cells' ability to grow on acetate medium (40). Consistent with this conclusion, it has been shown that *RTG2* is required for expression of the *ACO1* gene in glucose-repressed cells (41).

Here we report that expression of the genes encoding the first three steps of the TCA cycle leading to the synthesis of α-ketoglutarate, *CIT1*, *ACO1*, *IDH1*, and *IDH2*, switches between a dependence on the *HAP* genes in cells with robust mitochondrial function to the *RTG* genes in cells whose mitochondrial respiratory capacity has been reduced or eliminated. The remaining TCA cycle genes tested—all of which encode enzymes catalyzing steps downstream of isocitrate dehydrogenase—have no dependence on the *RTG* genes for their expression in either derepressed [*rho*$^+$] (respiratory competent) wild-type cells or [*rho*^{0}] petites. We analyze the control of *CIT1* expression in detail and show that it contains a functional R box in the 5′ flanking region of the gene that is required for Rtg1p-Rtg3p-dependent expression. We propose that the *RTG* control of genes encoding the first three enzymes of the TCA cycle leading to the synthesis of α-ketoglutarate is to ensure that sufficient glutamate is made for biosynthetic processes in cells with reduced respiratory capacity. Finally, we show that glutamate is a potent repressor of *RTG*-dependent gene expression, suggesting an important feedback regulation of glutamate synthesis. Like the *HAP* genes, which are responsible for a global control of gene expression in derepressed, respiratory competent cells, the *RTG* genes represent a major control pathway of gene expression in cells with reduced or compromised mitochondrial function.

MATERIALS AND METHODS

Growth media and growth conditions. Yeast strains were grown at 30°C in YP medium (1% yeast extract, 2% Bacto Peptone) with 2% raffinose (YPR) or 2 or 5% glucose (YPD or YP5%D, respectively); in YNB medium (0.67% yeast nitrogen base) supplemented with 1% Casamino Acids, leucine (30 mg/liter), lysine (30 mg/liter), and 2 or 5% dextrose (YNBcasD or YNBcas5%D), 2% raffinose (YNBcasR), 2% potassium acetate (YNBcasAce, pH adjusted to 5.5), or 2% glycerol (YNBcasGly); or in minimal YNB medium (0.67% yeast nitrogen base without Casamino Acids) supplemented with leucine (30 mg/liter), lysine (30 mg/liter), uracil (20 mg/liter), and 2 or 5% glucose (YNBD or YNB5%D) or 2% raffinose (YNBR), with or without sodium glutamate (concentrations indicated in the text and figures).

Strains. Yeast strains used in this study were PSY142 (*MAT*α *leu2 lys2 ura3* [*rho*$^+$]) and its derivatives constructed as follows. To construct the *rtg1*Δ derivative, a 674-bp *Hin*dIII-*Sst*I fragment of *RTG1* was replaced with a 1.2-kb *Xho*I-*Hin*dIII fragment of the *URA3* gene (18). Ura$^-$ derivatives were obtained by selection with 5-fluro-orotic acid. An *rtg2*Δ derivative was constructed by replacing a *Sal*I-*Xba*I fragment of *RTG2* with a 2.2-kb fragment of the *LEU2* gene, thus deleting codons 23 to 573 of *RTG2* (37). To construct an *rtg3*Δ derivative, codons 175 to 340 of *RTG3* were replaced with a 1.6-kb fragment of the *LEU2* gene (14). [*rho*^{0}] derivatives of these strains were generated by several passages of [*rho*$^+$] cells in YPD medium supplemented with 25 μg of ethidium bromide per ml. The *hap2*Δ deletion was obtained by replacing bp +44 to +686 of the *HAP2* gene with the kanMX4 cassette (42). The *hap2*Δ deletion was introduced into the wild-type and Δ*rtg1*, Δ*rtg2*, and Δ*rtg3* mutant strains. The *cit1*Δ and *cit2*Δ deletion mutants were described previously (18, 19, 40).

Plasmid constructs. The DNA region from −806 to +9 of *CIT1* was amplified by PCR. The forward primer contained a 5′ *Eco*RI restriction site and reverse primer contained a 5′ *Hin*dIII restriction site. The *CIT1* amplified product was inserted into the *Eco*RI-*Hin*dIII site of YIp356 to produce pCIT1-LacZ. pCIT1-LacZ was linearized by digestion with *Kpn*I and integrated into the genomic *CIT1* locus by standard yeast transformation procedures (32). The pACT1-LacZ plasmid was constructed similarly by inserting the PCR-amplified DNA region of *ACT1* from −667 to +9 into the *Hin*dIII-*Eco*RI site of YIp356R. The pACT1-LacZ plasmid was linearized by digestion with *Nco*I and integrated into the *URA3* locus. The pCIT2-LacZ and pDLD3-LacZ reporter gene constructs were described previously (4, 18). To make an R box mutant construct of pCIT1-LacZ, an internal pair of primers, 5′-gtacACGCGTTTTTTTCCGCCGCAG-3′ and 5′-gtacACGCGTCGCCTTTTAGCACAAAAATG-3′, (lowercase letters indicate plasmid sequence), was used to introduce two point mutations into the R box, changing GTCAC to GACGC. To construct pCIT1(UAS [upstream activation sequence])-CYC1-LacZ and pCIT1(R)(UAS)-CYC1-LacZ, a pair of primers, 5′-gact<u>aagctt</u>TGTATTTACCTTGCATTT-3′ and 5′-gact<u>ctcgag</u>GGAAAAGCTCCAAAGGG-3′, (underlining indicates restriction sites), was used to amplify the −400 to −260 region of *CIT1* in pCIT1-LacZ and pCIT1(R)-LacZ, respectively. The resulting amplified products were cleaved by *Hin*dIII and *Xho*I and inserted into the multiple cloning region of pWCJ100, which contains the minimal promoter of *CYC1* fused to *lacI-lacZ* coding sequence. Plasmid pWCJ100 is a centromere-based vector derived from pKM270. pACT1-CIT1 was constructed by fusing the *ACT1* region from −457 to −152 to the *CIT1* region from −170 to +1990 in plasmid YIp352. pACT1-CIT1 was linearized by digestion with *Apa*I and integrated into the *URA3* locus.

Electrophoretic mobility shift assay (EMSA). Whole-cell extracts were prepared as described previously (18) except that [*rho*^{0}] cells were used. Cells were grown in YNB5%D medium containing the necessary nutritional supplements and including 0.01% glutamic acid. A 140-bp DNA sequence from −400 to −260 of the *CIT1* gene amplified by PCR was used as a probe. The resulting PCR product was gel purified and end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The reaction mixture contained 25 mM Tris-HCl (pH 7.5), 5 mM MgCl, 0.125 mM EDTA, 300 mM KCl, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg of aprotinin per ml, 0.5 μg of leupeptin per ml, 1 ng of ^{32}P-labeled probe, 1 μg of salmon sperm DNA, and 40 μg of cell extract in a final volume of 20 μl. After 20 min of incubation at room temperature, the reaction mixture was applied to a 4% acrylamide gel (40:1 in 0.5× Tris-borate-EDTA buffer) prerun for 30 min and then run for 2.3 h at 4°C. The gel was dried and exposed to X-ray film.

Yeast transformation and β-galactosidase assays. Yeast cells were transformed as described by Chen et al. (5). Transformants carrying the desired plasmids were selected on YNBcasD plates. Liquid precultures were inoculated with a pool of several independent transformants and grown in YNBcasD medium. Cells were collected by centrifugation and diluted into YNBcas5%D or into YNBcasR medium and grown overnight to an optical density at 600 nm of ~2.0. Cells were collected by centrifugation, diluted into corresponding fresh medium, and collected at an optical density at 600 nm of ~0.8 after 9 h growth at 30°C. For glutamate repression analysis, YNB5%D and YNBR media were used. The preparation of cell extracts and β-galactosidase assays were carried out as described by Rose et al. (33). For each plasmid-strain combination, assays were conducted in triplicate and independent experiments were carried out two to three times.

RNA isolation and Northern blot analysis. Total yeast RNA was isolated from 50- to 200-ml logarithmic-phase cultures, fractionated on 1.2% agarose gels, transferred to Nytran Plus, and hybridized at 65°C with probes specific for transcripts of the *CIT2* and *ACT1* genes as previously described (14). The region from +263 to +1344 of *CIT1* was purified by cleaving a plasmid containing that sequence with *Cla*I and *Stu*I and used as probe for *CIT1* transcripts. The remaining probes were PCR amplified from selected coding regions of the genes of interest, using genomic DNA as a template. The PCR products were gel purified by using a GenecleanII kit from Bio 101 (Vista, Calif.). The regions amplified were −380 to +2855 of *ACO1*, −54 to +1237 of *IDH1*, −53 to +1279 of *IDH2*, −59 to +3226 of *KGD1*, −126 to +2171 of *SDH1*, and −63 to +1660 of *FUM1*. Hybridization signals were quantified with a Molecular Dynamics PhosphorImager.

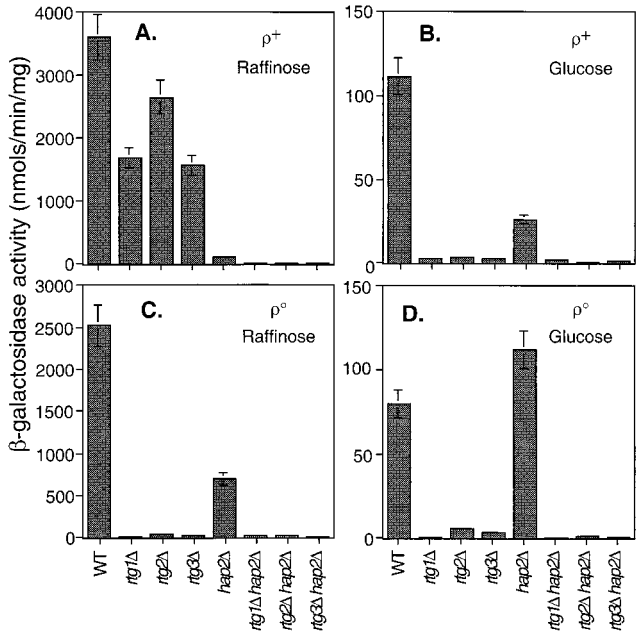


FIG. 1. Alternative dependence of *CIT1* expression on *RTG1*, *RTG2*, *RTG3*, and *HAP2*. β-Galactosidase assays were carried out to determine the activity of a *CIT1-lacZ* reporter gene in wild-type (WT) PSY142 [*rho*$^+$] and [*rho*^{0}] cells and various mutant derivatives of these strains as indicated. The bp −806 to +9 region of the *CIT1* gene was fused to the coding region of the *E. coli lacZ* gene, and the resulting construct was integrated at the chromosomal *CIT1* locus. For each strain grown on either raffinose (YPR) or glucose (YP5%D) medium, four independent transformants were pooled from mid-log-phase cultures and β-galactosidase assays on whole-cell extracts were carried out in triplicate as described in Materials and Methods.

RESULTS

Alternative dependence of expression of a *CIT1-lacZ* reporter gene on *HAP2* and *RTG1*, *RTG2*, and *RTG3*. To examine the role of the *RTG* genes in TCA cycle gene expression, we first investigated the regulation of *CIT1* expression by using a reporter gene construct in which 806 bp of the 5′ flanking region of *CIT1* was fused to the *Escherichia coli lacZ* gene. This construct was integrated into the *CIT1* locus of wild-type PSY142 [*rho*$^+$] and [*rho*^{0}] cells and into a *hap2*Δ and *rtg1*Δ, *rtg2*Δ, or *rtg3*Δ mutant derivatives of these strains. In agreement with the findings of Rosenkrantz et al. (34), the *CIT1-lacZ* reporter gene construct fully recapitulated the known glucose repression of the native *CIT1* gene, with more than a 15-fold decrease in β-galactosidase activity in extracts from [*rho*$^+$] cells grown on glucose compared with extracts from cells grown on raffinose, a nonrepressing carbon source (Fig. 1A and B). Reporter gene activity in [*rho*$^+$] *rtg*Δ cells was inhibited only modestly (25 to 50%) when those cells were grown on raffinose but was very strongly inhibited when those cells were grown on glucose. In derepressed [*rho*$^+$] cells, the *hap2*Δ mutation nearly abolished *CIT1* reporter gene expression; however, in glucose-repressed [*rho*$^+$] cells, significantly more reporter gene activity remained in the *hap2*Δ background. Finally, in [*rho*$^+$] cells grown on either raffinose or glucose, reporter gene activity was essentially eliminated in the *hap2*Δ *rtg*Δ double mutants. These results are fully consistent with the known requirement of the Hap2,3,4,5p complex for derepressed expression of the *CIT1* gene (34).

In contrast to these results, reporter gene expression was completely dependent on the *RTG* genes in [*rho*^{0}] petite cells, whether those petites were grown on raffinose or glucose (Fig. 1C and D). Although expression in [*rho*^{0}] cells still showed some dependence on *HAP2* when cells were grown on raffinose, *HAP2* was not required for expression in glucose-grown petite cells. Finally, as in [*rho*$^+$] cells, expression of the *CIT1-lacZ* reporter gene was eliminated in the *rtg*Δ *hap2*Δ double mutants. We conclude from these results that the *CIT1-lacZ* expression is largely dependent on the HAP system in cells with robust mitochondrial respiratory function but switches to a synergistic dependence on the RTG and HAP systems when respiratory function is reduced, as in glucose-repressed [*rho*$^+$] cells, or eliminated, as in [*rho*^{0}] petites. In the most severe case of reduced mitochondrial function—[*rho*^{0}] cells grown on glucose—*CIT1* expression is independent of the *HAP* system.

Glutamate auxotrophy. Previous studies showed that *rtg* mutant [*rho*$^+$] cells grown on minimal glucose medium were glutamate auxotrophs (18). Glutamate auxotrophy could arise from a block in the synthesis of α-ketoglutarate, the direct precursor of glutamic acid. The finding that *CIT1-lacZ* expression was largely independent of the *RTG* genes in [*rho*$^+$] cells grown on raffinose, but was strongly dependent on those genes in cells grown on glucose, raised the possibility that *rtg* mutant [*rho*$^+$] cells do not require glutamate for growth on minimal raffinose medium. We therefore compared the glutamate requirements for growth of wild-type [*rho*$^+$] and [*rho*^{0}] cells and their *rtg*Δ mutant derivatives on minimal raffinose or glucose medium. Figure 2A and Fig. 2B show that the *rtg*Δ mutant [*rho*^{0}] cells, in which *CIT1-lacZ* expression was strongly dependent on the *RTG* genes, were glutamate auxotrophs when grown on minimal raffinose medium. By contrast, the various *rtg*Δ [*rho*$^+$] mutant cells, in which *CIT1-lacZ* expression is largely independent of the *RTG* genes, were glutamate prototrophs on minimal raffinose medium. On minimal glucose medium, both the [*rho*^{0}] and [*rho*$^+$] *rtg*Δ mutant strains were glutamate auxotrophs (Fig. 2C and D), again consistent with the nearly complete dependence of *CIT1-lacZ* expression in those cells on the *RTG* genes.

The *CIT1* UAS contains a functional R box. A 10-bp DNA segment of *CIT1* from −367 to −358 has been shown to be important for glucose-repressed expression of the gene (34, 35). Notably, that 10-bp segment starts with GTCAC, an R box binding site for the Rtg1p-Rtg3p heterodimeric complex (14). To determine whether this R box confers the *RTG*-dependent expression of *CIT1*, we first constructed a *CIT1-lacZ* reporter gene in which a 140-bp fragment from −400 to −260 of the upstream region of *CIT1* containing both the R box site and the Hap2,3,4,5p binding site, CCAAT, was fused to a *CYC1* transcriptional start site linked to *lacZ* (Fig. 3A). The resultant *CIT1* UAS-*CYC1-lacZ* construct in a centromere plasmid was transformed into [*rho*$^+$] and [*rho*^{0}] cells and into *rtg3*Δ, *hap2*Δ, and *rtg3*Δ *hap2*Δ double-mutant derivatives of those strains. This reporter gene mimicked the profile of expression of the −806 bp *CIT1-lacZ* reporter gene: it was subject to glucose repression; expression was dependent on *RTG3* in [*rho*$^+$] and [*rho*^{0}] cells grown on glucose medium; and expression was dependent both on *HAP2* and on *RTG3* in [*rho*^{0}] cells grown on raffinose medium (Fig. 3B).

To determine whether the R box site is important for *RTG1,3*-dependent expression, two mutations were introduced into the R box of the *CIT1* UAS-*CYC1-lacZ* wild-type reporter construct (Fig. 3C), and the effects of those mutations were analyzed in [*rho*$^+$] and [*rho*^{0}] cells grown on raffinose or glucose medium. In [*rho*$^+$] cells grown on raffinose, the R box mutations reduced reporter gene expression less than threefold (Fig. 3D), whereas expression was reduced more than sixfold in [*rho*^{0}] cells grown on raffinose, and nearly ninefold in [*rho*^{0}] cells grown on glucose. These results suggest that the R

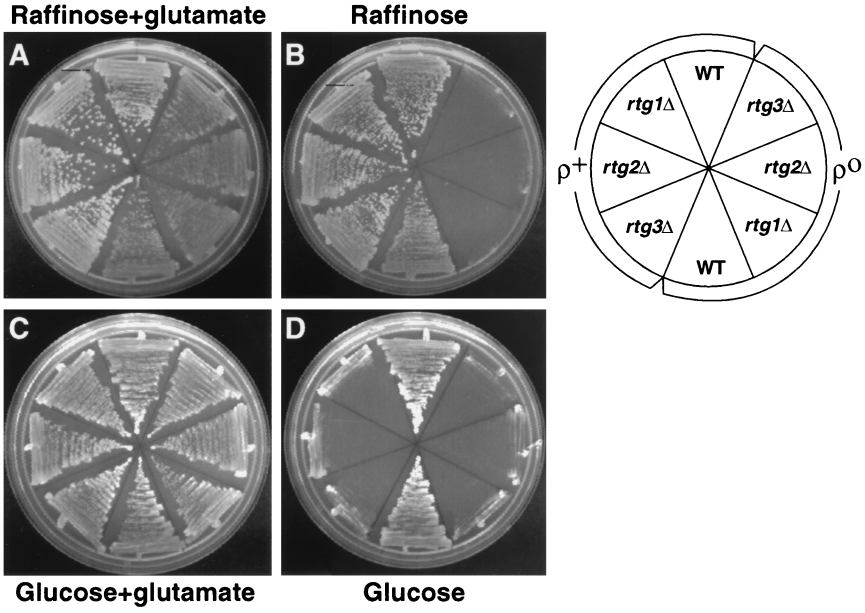


FIG. 2. Glutamate auxotrophy of *rtg* mutant cells coincides with *RTG*-dependent gene expression. Wild-type (WT) PSY142 [*rho*$^+$] and [*rho*^{0}] cells and *rtg1*Δ, *rtg2*Δ or *rtg3*Δ mutant derivatives of those strains were streaked on YNBR or YNBD medium with or without 0.02% glutamate. (A) YNBR plus glutamate; (B) YNBR alone; (C) YNBD plus glutamate; (D) YNBD. Only the [*rho*^{0}] mutant derivatives are glutamate auxotrophs in derepressed cells, whereas both [*rho*$^+$] and [*rho*^{0}] mutant derivatives are glutamate auxotrophs in glucose-repressed cells.

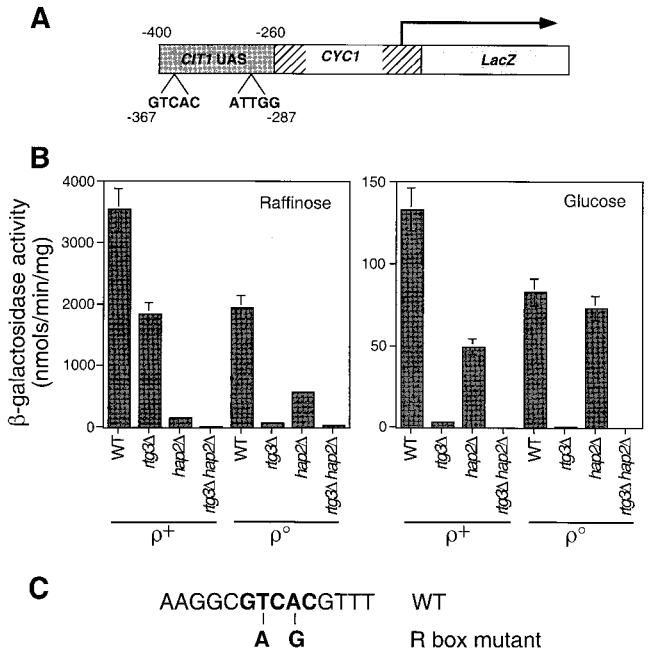
box in the *CIT1* UAS is an important *cis*-acting element for expression of *CIT1*, particularly in glucose-repressed [*rho*^{0}] cells in which mitochondrial function is most severely affected. These results are consistent with the differential dependence of *CIT1* expression on *RTG3* in [*rho*$^+$] and [*rho*^{0}] cells grown on repressing versus nonrepressing carbon sources.

To determine whether the Rtg1p-Rtg3p complex binds to the *CIT1* UAS, the 140-bp DNA fragment containing the R box was used as a probe in EMSAs with extracts from wild-type [*rho*$^+$] and *rtg*Δ mutant strains. With extracts from wild-type cells, two bands were detected by EMSA (Fig. 4, lane 2). However, when extracts from *rtg1*Δ or *rtg3*Δ strains were used, the fainter, lower band disappeared (Fig. 4, lanes 3 and 5). The faster-migrating band was still present when an extract from a *rtg2*Δ strain was used (Fig. 4, lane 4). This result is consistent with those of previous EMSAs with a *CIT2* UAS$_r$ probe showing that Rtg2p is not required for the binding of the Rtg1p-Rtg3p complex to R box sites (14, 18) and that Rtg2p is a cytoplasmic protein (38). Collectively, these data strongly suggest that the Rtg1p-Rtg3p complex regulates *CIT1* expression through interaction with the *CIT1* R box.

Glutamate represses *RTG* gene functions. Glutamate is a negative regulator of *CIT1* expression in glucose-repressed cells (15, 35). *ACO1*, whose expression requires Rtg2p in glucose-repressed cells (41), is also subject to glutamate repression (12). These observations raise the possibility that glutamate repression of *CIT1* expression is correlated with *CIT1*'s dependence on the *RTG* genes in glucose-repressed or respiratory deficient cells. To test this, we examined the effect of addition of glutamate to the growth medium on the expression of the −806 *CIT1-lacZ* reporter gene in [*rho*$^+$] and [*rho*^{0}] cells grown in medium with raffinose or glucose as the carbon source. In [*rho*$^+$] cells grown on raffinose, the addition of 0.01 or 0.2% glutamate to the medium resulted in only a partial inhibition of reporter gene expression (Fig. 5A), comparable to the inhibitory effect of the *rtg*Δ mutations in derepressed [*rho*$^+$] cells (Fig. 1). In contrast to these results, glutamate was a potent inhibitor of reporter gene expression in [*rho*$^+$] and [*rho*^{0}] cells grown on glucose and was slightly less effective as an inhibitor in [*rho*^{0}] cells grown on raffinose. Similar patterns of repression by glutamate were observed when we used a *CIT1* reporter gene containing the 140-bp *CIT1* UAS (data not shown). These findings support the conclusion that glutamate is a repressor of *RTG*-dependent *CIT1* expression.

The *RTG* genes are required for the expression the *CIT2* and *DLD3* genes in all strains and carbon sources that we have tested. If glutamate is a general negative regulator of *RTG*-dependent gene expression, then *CIT2* and *DLD3* expression should also be sensitive to glutamate repression independent of carbon source and the functional state of mitochondria. To test this, *CIT2-LacZ* and *DLD3-lacZ* reporter genes were introduced into [*rho*$^+$] and [*rho*^{0}] cells, which were then grown on either raffinose or glucose. Expression of both *CIT2-lacZ* and *DLD3-lacZ* was repressed by glutamate in the medium, and the extent of repression was independent of carbon source or the functional state of mitochondria (Fig. 5B). As a control, *lacZ* expression was placed under the control of the constitutive *ACT1* promoter, but expression of that fusion gene was not repressed by glutamate (data not shown). We conclude from these experiments that glutamate is a general repressor of *RTG*-dependent gene expression.

Constitutive expression of *CIT1* cannot rescue the phenotypes of *RTG* deletion mutants. Although the *RTG* genes are essential for *CIT2* expression, a *cit2*Δ mutant, unlike an *rtg*Δ mutant, is not a glutamate auxotroph, whereas the *cit1*Δ *cit2*Δ double mutant requires glutamate for growth (15). Given the finding that *CIT1* expression becomes dependent on the *RTG* genes in glucose-repressed or respiratory activity-deficient cells, we examined whether constitutive expression of *CIT1* would rescue the glutamate auxotrophy of *rtg*Δ mutant cells or their inability to grow on acetate medium. To these ends, we constructed a fusion gene in which *CIT1* expression was placed under the control of the constitutive promoter of the *ACT1* gene. The resulting plasmid, pACT1-CIT1, was transformed



β-galactosidase activity (nmols/min/mg)

	Raffinose		Glucose	
	ρ+	ρ°	ρ+	ρ°
WT	3541	1955	133	83
R box mutant	1303	292	23	9

FIG. 3. A functional R box in the *CIT1* UAS. (A) Diagram of a *CIT1* UAS-*CYC1-lacZ* construct in which a 140-bp fragment of the upstream region of *CIT1* from bp −400 to −260 was fused to the transcriptional start site of the *CYC1* gene and fused to the reading frame of the *E. coli lacZ* gene. Positions of the putative Rtg1p-Rtg3p R box binding site, GTCAC, and the Hap2,3,4,5p binding site, ATTGG, are indicated. (B) Wild-type (WT) PSY142 [*rho*$^+$] and [*rho*^{0}] cells and *rtg3*Δ, *hap2*Δ, and *rtg3*Δ *hap2*Δ derivatives were transformed with the *CIT1* UAS-*CYC1-lacZ* construct in a centromeric plasmid. Pools of 10 transformants of each were grown to mid-log phase on YNBcasR or YNBcas5%D medium, and β-galactosidase activity was determined in cell-free extracts. (C) Two mutations were introduced into the R box in the *CIT1* UAS-*CYC1-lacZ* construct as indicated in boldface and described in Materials and Methods. This construct was placed into a centromeric plasmid to yield pCIT1(R)(UAS)-CYC1-LacZ. (D) PSY142 [*rho*$^+$] and [*rho*^{0}] cells were transformed either with pCIT1(UAS)-CYC1-LacZ, containing the wild-type R box, or with pCIT1 (R)(UAS)-CYC1-LacZ, containing the mutant R box construct. Ten transformants of each were pooled and grown to mid-log phase in YNBcasR or YNBcas5%D medium, and β-galactosidase activities were measured in cell-free extracts. Standard errors from triplicate assays are <10%.

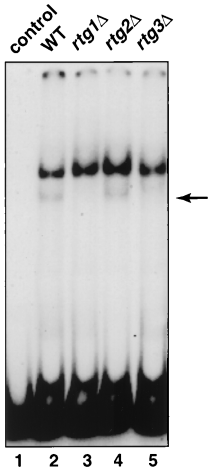


FIG. 4. The Rtg1p-Rtg3p complex binds to the *CIT1* UAS. Whole-cell extracts were prepared from wild-type (wt) [*rho*$^+$] PSY142 cells and from *rtg1*Δ, *rtg2*Δ, and *rtg3*Δ derivatives grown on YNB5%D medium supplemented with 0.01% glutamate. EMSA was carried out with a [γ-^{32}P]ATP-labeled 140-bp DNA probe of the *CIT1* UAS as described in Materials and Methods. The control lane 1 is the probe alone. The arrow indicates the gel-retarded band whose presence is dependent on Rtg1p and Rtg3p.

into a *cit1*Δ [*rho*$^+$] strain and into *rtg*Δ mutant derivatives of the wild-type [*rho*$^+$] strain. Controls included cells transformed with the vector alone, as well as a *cit2*Δ mutant strain. We then determined the ability of these strains to grow on YNBcasAce medium and on YNBD medium with or without glutamate supplementation. The constitutive expression of *CIT1* from pACT1-CIT1 was not able to restore glutamate auxotrophy to any of the *rtg*Δ mutant strains (Fig. 6A and B). Although all of these strains are respiratory competent, since they were able to grow on medium with glycerol as the sole carbon source (Fig. 6C), the *ACT1-CIT1* fusion gene restored growth to the *cit1*Δ mutant cells only when cells were grown on acetate medium (Fig. 6D). These data suggest that first, *CIT1* could be expressed from the *ACT1-CIT1* fusion gene sufficiently to restore acetate growth to a *cit1*Δ mutant, and second, the reduction in *CIT1* and *CIT2* expression in *rtg*Δ mutant cells is not solely responsible for their glutamate auxotrophy or inability of those cells to grow on acetate medium. Thus, other defects in the TCA cycle are likely to exist in these mutants.

Expression of *CIT1*, *ACO1*, *IDH1*, and *IDH2* requires the *RTG* genes in cells with dysfunctional mitochondria. To investigate further the relation between the *RTG* genes and expression of TCA cycle genes, we carried out Northern blot analysis to examine the relative expression of eight genes encoding proteins that function in the TCA cycle: *CIT1*, *ACO1*, *IDH1*, *IDH2*, *KGD1*, *SDH1*, *FUM1*, and *MDH1*. The transcript levels of these genes, normalized to the level of *ACT1* mRNA, were compared in [*rho*$^+$] and [*rho*^{0}] wild-type cells and in *rtg1*Δ, *rtg2*Δ, and *rtg3*Δ mutant derivatives of these strains grown in YPR medium. As a control, we also probed for *CIT2* transcripts, whose behavior has previously been well characterized in those strains. Only the *CIT2* gene showed a strong dependence on the *RTG* genes for expression in both [*rho*$^+$] and [*rho*^{0}] cells (Fig. 7). However, four of the TCA cycle genes, *CIT1*, *ACO1*, *IDH1*, and *IDH2*, which encode proteins catalyzing the first three steps of the TCA cycle from oxaloacetate to α-ketoglutarate, showed a dramatic dependence on the *RTG* genes for their expression in [*rho*^{0}] cells, but not in [*rho*$^+$] cells. In sharp contrast to the elevated expression of *CIT2* in [*rho*^{0}] cells, the transcript levels of these first four TCA cycle genes were about the same in [*rho*^{0}] and [*rho*$^+$] cells. Expression of the remaining TCA cycle genes tested, *KGD1*, *SDH1*, *FUM1*, and *MDH1*, was essentially unaffected by the *rtg*Δ mutations, and their expression in the wild-type [*rho*^{0}] strain was significantly less than in [*rho*$^+$] cells. Two transcripts that behaved identically were detected with the *SDH1* probe. The origin of these two transcripts has not been investigated further. The *HAP* genes are known to be required for expression of *KGD1*, *SDH1*, *FUM1*, and *MDH1* in derepressed [*rho*$^+$] cells (7, 12, 22, 31). Their reduced expression in derepressed [*rho*^{0}] cells provides the first indication to our knowledge that *HAP* functionality is reduced in derepressed, respiratory activity-deficient cells. From these experiments, we conclude that the

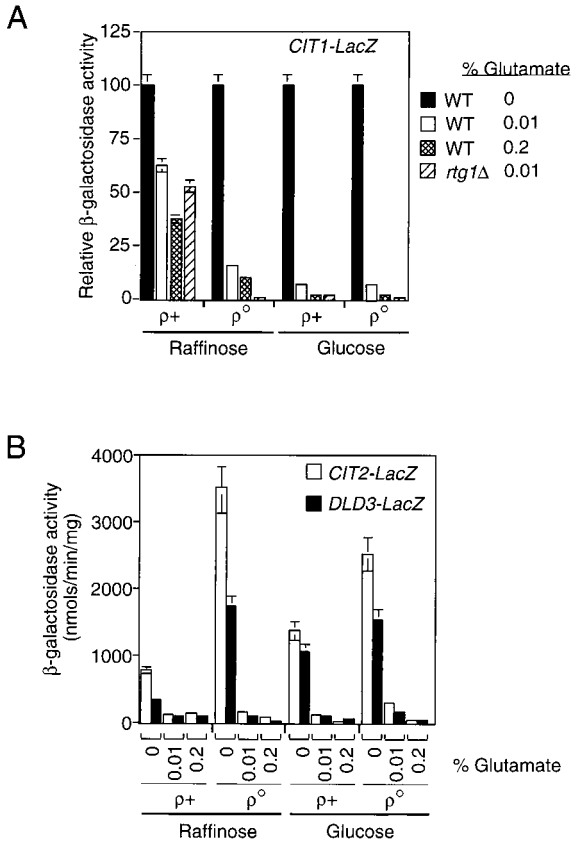


FIG. 5. Glutamate is a repressor of *RTG*-dependent gene expression. (A) PSY 142 [*rho*$^+$] and [*rho*^{0}] strains with an integrated copy of the −806 *CIT1-lacZ* reporter gene and an *rtg1*Δ derivative of those strains were grown in YNBR or YNB5%D medium as controls. Parallel cultures contained glutamate in the medium at the indicated concentrations. Whole-cell extracts were prepared, and β-galactosidase activities were determined. Data are expressed as β-galactosidase activities normalized to the value for the wild-type (WT) grown in the absence of glutamate. (B) Same as panel A except that wild-type [*rho*$^+$] and [*rho*^{0}] strains lacked the *CIT1* reporter gene and instead were transformed with centromere-based plasmids containing a *CIT2-lacZ* reporter gene (18) or a *DLD3* reporter gene in which the bp −500 region of *DLD3* was fused to *lacZ* (4).

expression of genes encoding proteins that function in the first three steps of the TCA cycle are under dual *HAP-RTG* control.

DISCUSSION

In an analysis of the *cis*-acting elements controlling expression of the mitochondrial citrate synthase gene, *CIT1*, Rosenkrantz et al. (34, 35) concluded that there were distinct upstream activation regions required for glucose-repressed and derepressed expression of the gene. In particular, the region from −367 to −348 was necessary for glucose-repressed expression, whereas the region from −291 and −273 was necessary for expression in derepressed cells. The latter is clearly identified as the *HAP* control region: it contains a consensus Hap2,3,4,5p binding site whose integrity is necessary for *CIT1* expression in derepressed cells, consistent with the known functionality of the Hap complex in cells with robust respiratory activity.

In the present study, we have shown that expression of *CIT1* becomes increasingly dependent on the *RTG* genes as mitochondrial respiratory function is reduced. The upstream region of *CIT1* identified by Rosenkrantz et al. (34, 35) as being necessary for glucose-repressed expression contains a single R box, GTCAC, which we have shown for the *CIT2* and *DLD3* genes is a binding site for the Rtg1p-Rtg3p, bHLH/Zip complex (4, 14). *CIT2* and *DLD3* each contain two closely spaced R boxes (in inverted orientation), both of which are necessary for maximal gene expression. Mutation of the single R box in the upstream region of *CIT1* shows that the R box is most important for *CIT1* expression in glucose-repressed cells, consistent with *CIT1*'s strong dependence on the *RTG* genes for its expression under glucose-repressed conditions. Together with the EMSAs showing that the Rtg1p-Rtg3p bind to a DNA probe containing the *CIT1* R box, these findings provide strong support for the conclusion that the *RTG* genes play an important role in the regulation of *CIT1* expression under conditions where *HAP*-dependent expression decreases.

We extended this dual *HAP-RTG* control of gene expression to include *ACO1*, *IDH1*, and *IDH2*. Velot et al. (41) reported that *ACO1* expression required *RTG2* specifically in glucose-repressed cells. Our studies confirm this finding and extend the control of *ACO1* expression as well as expression of the above-mentioned genes to include *RTG1* and *RTG3*. It was essential to test whether expression required all three of the *RTG* genes, because we do not yet know whether *RTG2* affects gene expression in pathways not involving *RTG1* or *RTG3*. We have inspected the 5′ flanking regions of *ACO1*, *IDH1*, and *IDH2* to see whether they contain R box sites. *ACO1* has two R boxes at −772 and −450. *IDH1* contains three R boxes at −355, −476, and −576, but the only consensus R box for *IDH2* evident from the database is one far upstream at −768. The functionality of these putative Rtg1p-Rtg3p binding sites and whether potential degenerate R box sites can serve as binding sites for Rtg1p-Rtg3p will have to be determined on a gene-by-gene basis. Finally, we found that expression of all of the other TCA cycle genes tested that encode proteins catalyzing steps of the cycle downstream of *IDH1* and *IDH2* was independent of the *RTG* genes in respiratory activity-deficient cells. Collectively, our findings show that the expression of genes encoding the first three steps of the TCA cycle from oxaloacetate to α-ketoglutarate come under increasing control of the *RTG* regulatory pathway as mitochondrial respiratory capacity is reduced. In this way, expression of those genes can be maintained to compensate for the loss of *HAP* gene control.

To evaluate the effects of the functional state of mitochondria on TCA cycle gene expression, it is worth emphasizing that the combination of strains and growth conditions that we chose have provided cells with a graded range of mitochondrial functions, from those with robust respiratory activity in derepressed [*rho*$^+$] cells to those with severely debilitated mitochondrial function in glucose-repressed [*rho*^{0}] cells. The striking observation was that the dependence of *CIT1* expression on the *HAP* and *RTG* genes followed this graded range of mitochondrial function: at the extremes, expression was largely *HAP* or *RTG* dependent, whereas in the middle, expression showed a synergistic dependence on the two systems. Although we do not know the precise details of how the control of expression of these TCA cycle genes is handed off from largely *HAP*-dependent expression in derepressed [*rho*$^+$] cells to *RTG*-dependent expression in glucose-repressed [*rho*^{0}] petites, it is not likely to be as a result of modulation of the level of the *RTG* gene products, since they are expressed constitutively in all cases that we have examined (14, 36, 37). Indeed, the *RTG* pathway appears to be controlled by an Rtg2p-dependent regulation of the nuclear localization of Rtg1p and Rtg3p (38). There is evidence that some of the components of the *HAP* complex are induced in cells growing on nonfermentable carbon sources, but how *HAP* activity is tied to the functional state of mitochondria is less clear.

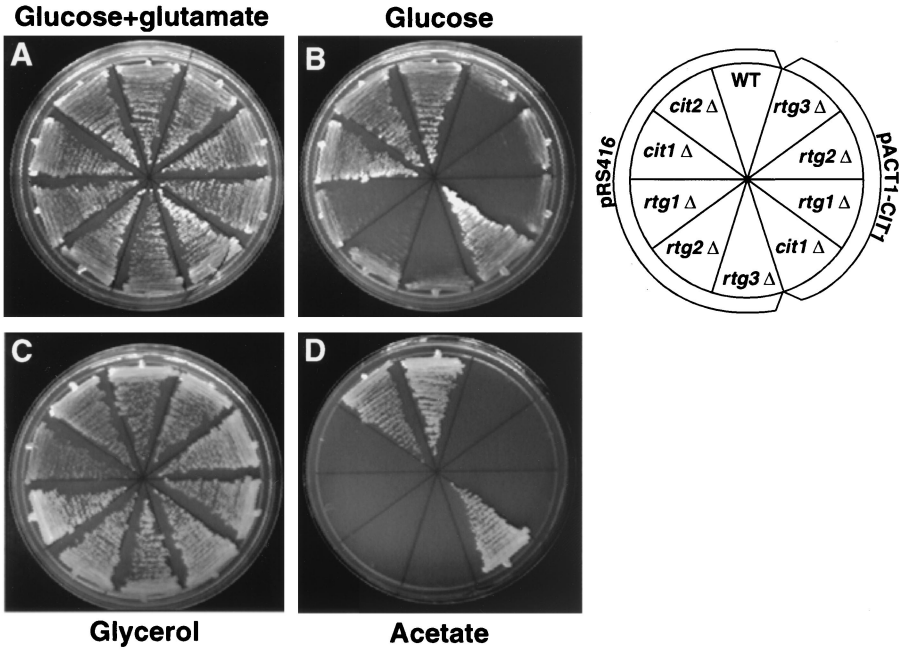


FIG. 6. Constitutive expression of *CIT1* fails to rescue the glutamate auxotrophy and acetate$^-$ phenotypes of *rtg* mutant cells. Wild-type (WT) PSY142 [*rho*$^+$] cells and *cit1*Δ, *cit2*Δ, *rtg1*Δ, *rtg2*Δ, or *rtg3*Δ derivatives were transformed with a centromere-based plasmid, pACT1-CIT1, in which *CIT1* expression was placed under the control of the constitutive *ACT1* promoter. As controls, these strains were also transformed with pRS416 alone. Cells were streaked on YNBD–0.02% glutamate (A), YNBD (B), YNBcasGly (C) or YNBcasAce (D) medium and grown for 2.5 to 3.5 days at 30°.

The retrograde response versus *RTG* control of TCA cycle gene expression. Our previous analysis of the *CIT2* and *DLD3* genes showed that their expression required the *RTG* genes, independent of the cell's respiratory state or the carbon source in the growth medium (4, 18, 19). A characteristic feature of *CIT2* and *DLD3* expression is their retrograde response, namely, a sharply elevated level of expression in cells with dysfunctional mitochondria. Based on the present findings, we can define two new patterns of gene regulation related to the mitochondrial state that apply to TCA cycle genes. The first is a dual dependence on the *HAP* and *RTG* genes, with an increasing reliance on the *RTG* genes for expression in cells whose mitochondrial respiratory function is reduced or eliminated. The overall levels of expression for those genes, *CIT1*, *ACO1*, *IDH1*, and *IDH2*, in contrast to retrograde response genes, are roughly the same in derepressed [*rho*$^+$] and [*rho*^{0}] cells. This combinatorial control between the *HAP* and *RTG* genes represents a heretofore unrecognized strategy by which cells regulate gene expression in response to carbon source and to changes in the functional state of mitochondria. The second pattern of control was evident from our Northern blot data showing that expression of TCA cycle genes functioning downstream of α-ketoglutarate, *KGD1*, *SDH1*, *FUM1*, and *MDH1*, are all down-regulated in [*rho*^{0}] petites.

We can consider three general pathways used by yeast cells to modulate expression of genes related to mitochondrial oxidative metabolism. The first is responsive to carbon source and is controlled by the Hap2,3,4,5p complex (10, 11, 24, 26, 27, 30, 43). Second are the oxygen-sensing pathways exemplified by control via the positive regulator, Hap1p (6, 29), and the negative regulator, Rox1p, a heme-dependent repressor of hypoxic genes (20, 21, 44). Hap1p is a transcriptional activator that, together with heme, responds to oxygen levels to regulate expression of an assortment of genes that function in electron transport, oxidative stress, and heme, sterol, and unsaturated fatty acid biosynthesis (reviewed by Kwast et al. [17]). The *RTG* genes can now be included as an additional pathway of gene regulation that monitors the functional state of mitochondria. As mitochondrial respiratory functions are compromised, the *RTG* system takes over responsibility for expression of some, otherwise *HAP*-dependent genes as described here and elevates the expression of other genes such as *CIT2* (19) to compensate for the mitochondrial defects.

A central role for glutamate. A number of previous studies had shown that glutamate is an inhibitor of expression of some TCA cycle genes as well as *CIT2* (13, 15, 35, 41), but the regulatory targets affected by glutamate were unknown. It is now clear that glutamate inhibition occurs via *RTG*-dependent gene expression, which we suggest is a reflection of a negative feedback loop that regulates glutamate levels in cells with compromised mitochondrial function. In yeast, there are three known pathways for glutamate synthesis (1, 9, 25): two glutamic dehydrogenase isozymes encoded by *GDH1* and *GDH3* and glutamine synthetase encoded by *GLT1*. All three pathways use α-ketoglutarate as a common precursor of glutamate, which itself is a precursor for the synthesis of other amino acids and nucleotides. As illustrated in the model shown in Fig. 8, the *RTG* pathway could be activated to ensure that (i) there is sufficient synthesis of α-ketoglutarate for glutamate synthesis when the *HAP* system is downregulated, as in respiratory deficient cells, and (ii) anaplerotic pathways and gluconeogenesis are adequately maintained as a result of increased supply of intermediates to the TCA cycle, particularly succinate, from the glyoxylate cycle. The dramatic increase in *CIT2* expression in respiratory activity-deficient cells could effectively increase the flux of carbon through the glyoxylate cycle to provide the net carbon needed for anabolic pathways.

Glutamate appears to be a key player in the *RTG* pathways. When supplemented in the growth medium, glutamate is a potent repressor of *RTG*-dependent gene expression, suggest-

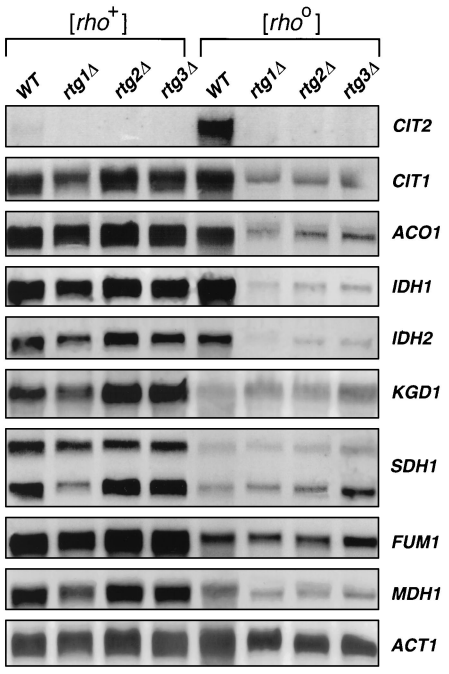


FIG. 7. Northern blot analysis of TCA cycle gene expression. Total RNA was prepared from PSY142 [*rho*$^+$] and [*rho*^{0}] wild-type (WT) strains and their *rtg1*Δ, *rtg2*Δ, and *rtg3*Δ derivatives grown to mid-log phase on YPR medium. Blots were probed for each of the indicated genes as described in Materials and Methods. RNA loads were normalized to the level of transcripts of the *ACT1* gene. *CIT2* transcripts were also analyzed as a control.

ing that if the *RTG* pathway indeed functions to maintain adequate levels of α-ketoglutarate for glutamate biosynthesis, it is subject to a strong glutamate feedback loop via transcriptional control, where expression of genes encoding the first three enzymes of the TCA cycle that lead to the synthesis of α-ketoglutarate are repressed by glutamate. Considering the findings to date, we are inclined to speculate that the intracellular level of glutamate or its direct precursor α-ketoglutarate might be a key signal for regulation of the *RTG*-dependent pathways.

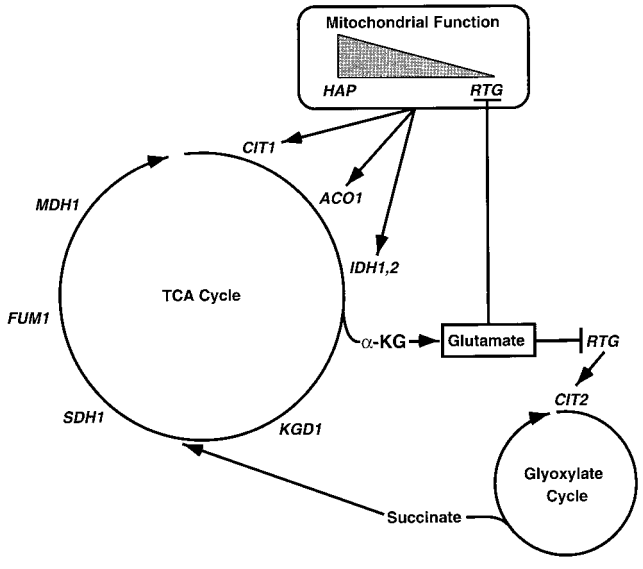


FIG. 8. Model for the role of the *RTG* genes in the control of expression of TCA and glyoxylate cycle genes. As mitochondrial function decreases (shaded triangle), the expression of *CIT1*, *ACO1*, *IDH1*, and *IDH2* becomes increasingly dependent on the *RTG* genes and less dependent on the *HAP* genes. Glutamate is shown as a feedback regulator of *RTG*-dependent pathways of gene expression. α-KG, α-ketoglutarate.

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