ATO3 Encoding a Putative Outward Ammonium Transporter Is an RTG-independent Retrograde Responsive Gene Regulated by GCN4 and the Ssy1-Ptr3-Ssy5 Amino Acid Sensor System*

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Respiratory deficient yeast cells such as ρo petites activate an inter-organelle signaling pathway called retrograde regulation. This results in changes in the expression of a subset of nuclear genes leading to major reconfigurations of metabolism that enable cells to adapt to the respiratory deficient state. Previous studies have focused on the role of three positive regulatory factors in the retrograde pathway, Rtg1p, Rtg2p, and Rtg3p, which are essential for both basal and elevated expressions of some, but not all, retrograde responsive genes. Here we characterize the retrograde regulation of one of those genes, ATO3, whose elevated expression in ρo petites is largely independent of RTG gene function. ATO3 encodes a member of the YaaH family of proteins that is a putative outward ammonium transporter. We show that Ato3p-green fluorescent protein is preferentially localized to the plasma membrane of mother cells. ρo petites express more Ato3p-green fluorescent protein in their plasma membrane than do ρ+ cells, consistent with the elevated level of ATO3 transcripts in ρo cells. We find that ATO3 expression has two levels of control, both of which are connected to amino acid sensing and regulation. The first involves GCN4, which is required for the bulk of ATO3 expression. The second involves the Ssy1-Ptr3-Ssy5 amino acid sensor system, which is preferentially required for elevated ATO3 expression in ρo cells. We propose that ATO3 is induced in ρo cells to eliminate the excess ammonia that arises because of a potential defect in ammonia assimilation in those cells.

Cells reconfigure their pattern of gene expression to adjust to changes in nutrient availability or in response to stress. In cells of Saccharomyces cerevisiae, for example, dramatic changes in gene expression occur during the diauxic shift (1), which is the switch from the utilization of glucose as carbon source to the utilization of ethanol, produced from glycolysis. Yeast cells also modulate the expression of genes in response to mitochondrial dysfunctions (2–4). This inter-organelle 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.

The expression of genes such as CIT2, DLD3, and PDH1 is dramatically up-regulated in cells with dysfunctional mitochondria (2, 4, 5), for instance, in ρo petites, which are respiratory deficient cells that lack mitochondrial DNA. Regulation of both basal and elevated expression of these genes in cells with dysfunctional mitochondria is largely dependent on the RTG genes, RTG1–3. RTG1 and RTG3 encode basic helix-loop-helix/leucine zipper (bHLH/Zip) transcription factors, Rtg1p and Rtg3p, that bind as a heterodimer to R box sites (GTGAC) in the promoter of genes such as CIT2 (6). Rtg2p, a novel cytoplasmic protein with an N-terminal ATP binding domain, transduces mitochondrial signals to regulate the cytoplasmic-to-nuclear translocation of Rtg1p and Rtg3p in response to the functional state of mitochondria (7). That nuclear translocation step is blocked by Mks1p, a negative regulator of RTG-dependent gene expression (8–10). Rtg2p acts to positively regulate the RTG pathway by sequestering Mks1p in an inactive form (11). In cells with compromised or dysfunctional mitochondria, the expression of CIT1, ACO1, IDH, and IDH2, which encode enzymes catalyzing the first three steps of the tricarboxylic acid cycle that produce α-ketoglutarate, the precursor to glutamate, is also under the control of the RTG genes (12). Thus, in ρo mutant cells with compromised or dysfunctional mitochondria, not only is there a loss in expression of genes such as CIT2, but those cells are also glutamate auxotrophs (4, 12). Moreover, glutamate is a potent repressor of RTG-dependent gene expression (12), underscoring the important role that the RTG pathway plays in glutamate homeostasis. The RTG regulatory circuit has also been shown to be activated in cells in which the target of rapamycin (TOR) pathway is inhibited by treating cells with the immunosuppressant rapamycin (13), a finding that suggests that the retrograde pathway integrates carbohydrate and nitrogen metabolism.

In a previous study (2) aimed at identifying genes whose expression is altered as a result of mitochondrial dysfunction, we identified >40 genes in the yeast transcriptome whose expression was consistently elevated by at least 2-fold in ρo versus ρ+ isochromosomal cultures grown on the fermentable but non-repressing carbon source, raffinose. In that study, we also determined the effects of inactivation of the RTG genes. These experiments revealed a subset of genes whose elevated expression in ρo petites appeared to be independent of the RTG genes, suggesting the existence of other retrograde signaling pathways activated by mitochondrial dysfunctions.

Here we characterize a retrograde responsive gene, ATO3 (YDR384c), whose elevated expression in ρo petite cells is largely independent of the RTG genes. ATO3 encodes a member of the YaaH family of proteins with six transmembrane domains that has been proposed to be an outward ammonium transporter (14). ATO3 expression is dependent on Gcn4p, a transcriptional activator of the general amino acid control.
pathway (15), and its retrograde response is largely dependent on the Ssy1-Ptr3-Ssy5 (SPS)\(^1\) amino acid sensing pathway. We propose that respiratory deficient cells have a stoichiometric imbalance between \(\alpha\)-ketoglutarate and ammonium, so that the elevated expression of ATO3 in those cells is intended to overcome the potential toxic effects of excess ammonium.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions**—The *S. cerevisiae* strains used in this study were derivatives of PSY142 (MATa leu2 lys2 ura3), S288c (MATa ura3-52), and PLY126 (MATa ura3-52 lys2Δ201). \(\phi\) r promoters were obtained by growth of \(\phi\) r cells on rich dextrose medium containing 25 \(\mu\)g/ml ethidium bromide. Each \(\phi\) r derivative was stained with 4′,6-diamino-2-phenylindole to verify that no mitochondrial DNA were present.

Deletion of *GCN4* was performed by PCR amplification of the KanMX4 deletion module from the commercially available Research Genetics strain (Invitrogen). The genomic DNA used for PCR amplification was isolated from kanamycin-resistant spores obtained after sporulation of *gcn4-KanMX4/GCN4* cells. The PCR products were used to replace the *GCN4* wild-type gene in \(\phi\) r and \(\phi\) r isolates of strain PSY142, and the replacement was confirmed by PCR analysis.

Cells were grown at 30 °C on the following media as indicated in the text under "Results" and in all of the figure legends: rich YP medium (1% yeast extract and 2% Bacto-peptone) containing 2% raffinose (YPY); YNB medium (0.67% yeast nitrogen base without amino acids) (1% yeast extract and 2% Bacto-peptone) containing 2% raffinose (YNB); and 2% raffinose (YNB). The primers used to amplify each fragment were as follows: 5′-AAGCTT CCA TAC GGA AGA GTC TCA-3′ for fragment I (from *MATa ura3*); 5′-AAGCTT CAG TAG CAT TCT TGG-3′ for fragment II (from *MATa ura3*); and 5′-AAGCTT CCA TAC GGA AGA GTC TCA-3′ for fragment III (from *MATa ura3*). The primers contained a HindIII restriction site, and all the reverse primers contained a BamHI restriction site (12). The primers used to amplify each fragment were as follows: 5′-AAGCTT CCA TAC GGA AGA GTC TCA-3′ for fragment I (from *MATa ura3*); 5′-AAGCTT CAG TAG CAT TCT TGG-3′ for fragment II (from *MATa ura3*); and 5′-AAGCTT CCA TAC GGA AGA GTC TCA-3′ for fragment III (from *MATa ura3*).

**Plasmid Constructs**—Standard techniques were used for the construction, amplification, and manipulation of plasmids. An ATO3-lacZ reporter gene was constructed by PCR amplification of a selected ATO3 promoter region, using as forward primer 5′-AAGCTT CCA TAC GGA AGA GTC TCA-3′ and as reverse primer 5′-AAGCTT CAG TAG CAT TCT TGG-3′. The primers used to amplify each fragment were as follows: 5′-AAGCTT CCA TAC GGA AGA GTC TCA-3′ for fragment I (from *MATa ura3*); 5′-AAGCTT CAG TAG CAT TCT TGG-3′ for fragment II (from *MATa ura3*); and 5′-AAGCTT CCA TAC GGA AGA GTC TCA-3′ for fragment III (from *MATa ura3*). The fragments used for the promoter dissection were obtained by PCR amplification of the following DNA regions of the ATO3 promoter: fragment I (from -999 to -593 bp); fragment II (from -611 to -285 bp); and fragment III (from -553 to -36 bp). The fragments were cloned into pWCV100 containing the CYC1-TATA transcriptional start site (12). The primers used to amplify each fragment were as follows: 5′-GTCGAG CCG TAT TGT TGT TAT GAT CTA CTA-3′ and 5′-GTCGAG CCG TAT TGT TGT TAT GAT CTA CTA-3′ for fragment I; 5′-GTCGAG CCG TAT TGT TGT TAT GAT CTA CTA-3′ and 5′-GTCGAG CCG TAT TGT TGT TAT GAT CTA CTA-3′ for fragment II; and 5′-GTCGAG CCG TAT TGT TGT TAT GAT CTA CTA-3′ and 5′-GTCGAG CCG TAT TGT TGT TAT GAT CTA CTA-3′ for fragment III. The all the fragments were cloned into the centromeric plasmid, pWEJ, derived from pKM270 (5).

**RESULTS**

**The Elevated Expression of ATO3 and DIP5 in \(\phi\) r**

**Petites Is Independent of the RTG Genes**—To verify that some retrograde responsive genes are RTG-independent (2), we examined the expression of two candidate genes, DIP5 and ATO3, by Northern blot analysis in wild-type \(\phi\) r and \(\phi\) r cells. By comparison, we also examined the expression of CIT2, which is a prototypical retrograde responsive gene whose expression is strictly dependent on the RTG genes (4, 6). Fig. 1A shows the characteristic increase of CIT2 transcripts in \(\phi\) r cells and the complete dependence of CIT2 expression on RTG1–3. Like CIT2, the expression of both DIP5 and ATO3 was significantly elevated in \(\phi\) r cells, indicating that they are also retrograde responsive genes. However, in striking contrast to CIT2, inactivation of RTG2 had no effect on the level of DIP5 and ATO3 transcripts in \(\phi\) r cells, and there was only a modest (−50%) reduction in the level of those transcripts in *rtg1Δ rtg3Δ* \(\phi\) r cells. By contrast, CIT2 expression is inhibited >99% in *rtg1Δ rtg3Δ* cells. In the subsequent experiments described here, we have focused on the analysis of ATO3 expression as an example of a retrograde responsive gene whose expression is largely independent of the RTG genes.

To verify that the changes we observed in ATO3 mRNA levels were transcriptionally controlled, we fused the 5′ flanking region of ATO3 plus the first four amino acids (from positions −981 to 12) in-frame with the *Escherichia coli lacZ* gene. These constructs on centromeric plasmids were transformed into \(\phi\) r or \(\phi\) r wild-type cells and *rtg1Δ rtg3Δ* cells. The DIP5 and ATO3 probes were hybridized at 65 °C with probes specific for transcripts of DIP5 and ATO3. The DIP5 and ATO3 probes were amplified by PCR from selected coding regions of the reported genes using genomic DNA as template. The PCR products were gel-purified using a Qiaquick kit. The CIT2 and ACT1 probes were prepared as described by Jia et al. (16). RNA loads were normalized to the level of ACT1 transcripts. Hybridization signals were quantified with an Aersham Biosciences PhosphorImager.

**Fluorescence Microscopy**—Mid-log phase cells were observed using a Leica microscope (model DMRXE) equipped with an HBO 100 watt/2 mercury arc lamp and a 100× Plan-Apochromatic objective lens. Differential interference contrast and green fluorescent protein were visualized using filter sets as described in Bateman et al. (16).

**Yeast Transformation and \(\beta\)-Galactosidase Assay**—Yeast cells were transformed as described by Chen et al. (17). Transforms carrying the desired plasmids or plasmid pairs were selected on a selective medium. Liquid pre-cultures were inoculated with a pool of several independent transformants and grown on a selective medium. The cultures were inoculated at a low density (OD550 = 0.011), grown overnight at 30 °C, and collected at OD550 = 0.6. Cell extracts and \(\beta\)-galactosidase assays were carried out as described by Rose et al. (18). For each plasmid-strain combination, assays were conducted in triplicate, and independent experiments were carried out 2–3 times.

**RNA Isolation and Northern Blot Analysis**—Total yeast RNA was isolated as described (19) from 50 or 250 ml of mid-log phase liquid cultures (OD550 = 0.8–1.0). Samples (10–15 μg) were fractionated on 1.2% formaldehyde gels, transferred to Nytran Plus filters (Schleicher & Schuell), and hybridized at 65 °C with probes specific for transcripts of CIT2, ACT1, and DIP5. The CIT2, ACT1, and DIP5 probes were amplified by PCR from selected coding regions of the reported genes using genomic DNA as template. The PCR products were gel-purified using a Qiaquick kit. The CIT2 and ACT1 probes were prepared as described by Jia et al. (16). RNA loads were normalized to the level of ACT1 transcripts. Hybridization signals were quantified with an Aersham Biosciences PhosphorImager.

**TOTAL RNA**—Total RNA was isolated from kanamycin-resistant spores obtained after transformation of *psd1-1* strains used in the current studies. Yeast cells were grown at a low density (OD600 = 0.6). Cell extracts and \(\beta\)-galactosidase assays were carried out as described by Rose et al. (18). For each plasmid-strain combination, assays were conducted in triplicate, and independent experiments were carried out 2–3 times.

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1. The abbreviations used are: SPS, Ssy1-Ptr3-Ssy5; GFP, green fluorescent protein.
ATO3 Is an RTG-independent Retrograde Responsive Gene

**Fig. 1.** RTG-independent retrograde responsive genes. A, Northern blot analysis. Total RNA was isolated from wild-type (WT) PSY142 \( \rho^o \) and \( \rho^+ \) cells and from \( \text{rtg}2 \Delta \) and \( \text{rtg}1 \Delta \text{rtg}3 \Delta \) (\( \text{rtg}1 \Delta \text{rtg}3 \Delta \) \( \rho^o \)) derivatives grown to mid-log phase on YPR medium. The blots were hybridized with probes specific for each of the indicated genes. RNA loads were normalized to the level of transcripts of the act1 gene. B, activation of an ATO3-lacZ reporter gene in \( \rho^o \) cells is not affected by an \( \text{rtg}2 \Delta \) mutation and is only partially reduced in an \( \text{rtg}1 \Delta \text{rtg}3 \Delta \) double mutant. Wild-type PSY142 \( \rho^+ \) and \( \rho^o \) cells and \( \text{rtg}2 \Delta \) and \( \text{rtg}1 \Delta \text{rtg}3 \Delta \) \( \rho^o \) derivatives were transformed with the ATO3 promoter fused to the lacZ reporter gene on a centromeric plasmid (see "Experimental Procedures"). Cultures were inoculated with a pool of 10–15 transformants each and grown to early log phase on YNBRcas medium. \( \beta \)-Galactosidase assays were carried out in triplicate on cell-free extracts.

**Fig. 2.** Ato3p-GFP fusion protein localizes to the plasma membrane. Wild-type PSY142 \( \rho^+ \) and \( \rho^o \) cells were transformed with a centromeric plasmid containing the ATO3 5' flanking sequences and coding region tagged at its C terminus with GFP. Transformants were grown in YNBRcas medium and collected at mid-log phase for microscopic analysis. Representative \( \rho^+ \) and \( \rho^o \) cells in the population are shown. DIC, differential interference contrast.

**Fig. 3.** Identification of a control region in the ATO3 promoter. A, the ATO3 promoter region was divided into three fragments, indicated as I, II, and III. Each fragment was inserted in front of the CYC1 transcriptional start site fused to the lacZ gene, and these constructs were inserted into centromeric plasmids. B, region II of the 5' flanking sequence of ATO3 promoter is sufficient to activate reporter gene expression. Wild-type PSY142 \( \rho^+ \) and \( \rho^o \) cells were transformed, respectively, with constructs I, II, and III, shown in panel A. Pools of 10–15 transformants each were grown to early log phase on YNBRcas medium. \( \beta \)-Galactosidase assays were carried in triplicate on cell-free extracts.

**Dissection of ATO3 Promoter and Identification of a Gcn4p Binding Site**—To identify the cis and trans regulatory elements that control the expression of ATO3, we first sought to identify regions of the 5' flanking sequences of ATO3 that would support reporter gene activity and show a retrograde response. To these ends, we divided the 5' flanking regions of ATO3 into three fragments (Fig. 3A), namely I (from −999 to −593), II (from −611 to −285), and III (from −353 to −36), and inserted these in front of the CYC1 transcriptional start site fused to the lacZ gene (Fig. 3A). These constructs on CEN plasmids were used to transform PSY142 \( \rho^+ \) and \( \rho^o \) cells, and \( \beta \)-galactosidase activity was measured in cell-free extracts obtained from pools of transformants grown on YNBRcas medium. The promoter dissection revealed clearly that the region contained within fragment II from −611 to −285 was sufficient to activate reporter gene expression and support its elevated expression in \( \rho^o \).
centromeric plasmid. Transcriptional start site fused to the lacZ construct was transformed into PSY142 constructs A, B, and C, and A transformed with construct II, described in the legend to Fig. 3.

Wild-type PSY142, having the A/H9267 type PSY142 promoter, shows the highest activity for reporter gene expression. Wild-type PSY142 region B, containing a GCN4p binding site induces reporter gene expression on YNBR medium. Wild-type PSY142 region B, containing a GCN4p binding site induces reporter gene expression. However, in gcn4Δ cells, the fold inhibition of ATO3 expression was comparable for ρ+ and ρ- cells, indicated in Fig. 5 as the ρ'/ρ- expression ratio, showing that the retrograde response of ATO3 was relatively unaffected by the gcn4Δ mutation. Thus, although Gcn4p contributes to ATO3 expression, it is not required for the retrograde responsiveness of this gene.

ATO3 Expression Is Modulated by SSY1—ATO3 was reported to be one of a number of genes whose expression is dependent on the SPS sensing system for extracellular amino acids (20, 21). This would be consistent with the finding that ammonia production is decreased in cells that are defective in the SPS system (22). Forsberg et al. (21) also found that the expression of a significant number of SPS-dependent genes in prototrophic cells was either derepressed or repressed by the addition of leucine to the medium. We were therefore interested in examining the effects of inactivation of the upstream component of the SPS system, SSY1, on both the expression and the retrograde response of ATO3. Although Forsberg et al. (21) did not find that ATO3 expression was responsive to leucine, we nevertheless tested the effects of leucine on ATO3-lacZ expression in PLY126 ρ+ and ρ- wild-type and ssy1Δ cells. Both wild-type and ssy1Δ ρ+ and ρ- derivatives of PLY126 were grown in YNB medium in the presence or absence of 0.05% leucine, and ATO3 reporter gene activity was assayed in cell extracts. As shown in Fig. 6, deletion of SSY1 has no effect on ATO3-lacZ reporter gene activity in ρ- cells grown in medium lacking leucine. By contrast, the elevated level of reporter gene activity in ρ- cells grown in the same medium was inhibited by ~60% as a result of inactivation of SSY1, indicating that the SPS sensor system plays a significant role in the

stein et al. (2) found that some Gcn4p-responsive genes were elevated in ρ- petite cells. Therefore, to test the functionality of the Gcn4p binding site in fragment B, PSY142 ρ+ and ρ- cells transformed with the CEN plasmid containing fragment B were grown in minimal YNBR medium (supplemented only with leucine and lysine to meet the nutritional requirements of this strain) so as to induce the general amino acid control pathway. Under these growth conditions, fragment B clearly had the highest activity in both ρ+ and ρ- cells. The activities in ρ- cells were comparable for fragment B and the full-length fragment II, and, in ρ- cells, the activity was roughly 70% of that of the full-length fragment II (Fig. 4C).

Gcn4p Is Required for Full ATO3 Expression but Not for Its Retrograde Response—To show directly that Gcn4p plays a role in ATO3 expression, we inactivated the GCN4 gene and tested the effects on ATO3 transcript levels in PSY142 ρ+ and ρ- cells. Fig. 5 shows that the level of ATO3 transcripts was reduced by 2.4- and 3.7-fold, respectively, in ρ+ and ρ- gcn4Δ cells relative to the wild-type control, indicating that Gcn4p is a regulator of ATO3 expression. However, in gcn4Δ cells, the fold inhibition of ATO3 expression was comparable for ρ+ and ρ- cells, indicated in Fig. 5 as the ρ'/ρ- expression ratio, showing that the retrograde response of ATO3 was relatively unaffected by the gcn4Δ mutation. Thus, although Gcn4p contributes to ATO3 expression, it is not required for the retrograde responsiveness of this gene.

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Regulation of the retrograde response pathway in yeast has been best characterized by the activity of three positive regulatory factors, Rtg1p, Rtg2p, and Rtg3p. For CIT2, a prototypical retrograde responsive gene, both its basal and elevated expression in cells with compromised or dysfunctional mitochondria is dependent on Rtg1p, Rtg2p, and Rtg3p (6). In the present study, we have described a retrograde responsive gene, ATO3, whose expression, like that of CIT2, is dramatically increased in respiratory deficient \( \rho^- \) petite cells but is largely independent of Rtg1p, Rtg2p, and Rtg3p. We showed that expression of ATO3 is regulated in part by the transcriptional activator Gcn4p and the SPS amino acid-sensing pathway. Ato3p has been suggested to be an outward ammonia transporter that contributes to the periodic production of ammonia as a means of signaling among yeast colonies (14). Ato3p is predicted to have six transmembrane domains and, thus, as a putative outward ammonia transporter, should be localized to the plasma membrane. This was confirmed by a microscopic analysis of cells expressing a GFP-tagged derivative of Ato3p. We observed a higher level of plasma membrane-localized Ato3p-GFP in \( \rho^- \) cells compared with their \( \rho^+ \) counterparts, consistent with the increased level of ATO3 transcripts in \( \rho^- \) cells. Ato3p-GFP also appeared to be preferentially localized to the plasma membrane of mother cells, as immature daughter buds contained significantly less signal. The functional significance and mechanism of the preferential accumulation of Ato3p to the plasma membrane of mother cells in log phase cultures remains to be established.

We have described two levels of control of ATO3 expression, both of which are connected to amino acid sensing and regulation. First, we found that ATO3 expression is regulated by Gcn4p, the transcriptional activator of the general amino acid control response pathway (15). Inactivation of GGC4 led to a severe reduction in the overall level of ATO3 expression in both \( \rho^- \) and \( \rho^+ \) cells, consistent with the presence of a potential Gcn4p binding site in the 5' flanking region of the gene. Despite its lowered expression in gcn4Δ cells, the fold induction of ATO3 transcripts in \( \rho^- \) versus \( \rho^+ \) gcn4Δ cells was comparable with that detected in wild-type cells. Second, we observed that ATO3 expression is also controlled by the SPS sensor system consisting of a plasma membrane protein, Ssy1p, and the peripheral membrane proteins Pto6p and Ssy5p. The latter two proteins are believed to interact with a cytoplasmic N-terminal extension of Ssy1p and, together, function as a signal transduction cascade for the sensing of external amino acids (20, 21, 23, 24). That the SPS amino acid sensor system plays a role in the regulation of ATO3 expression was suggested previously from a genome-wide transcriptional survey of the effects of the inactivation of SSY1 (21). In our current study we find that the inactivation of SSY1 had no effect on the expression of ATO3 reporter gene expression in \( \rho^- \) cells, but it resulted in a ~60% reduction of expression in \( \rho^+ \) petites. In other words, in contrast to the results of inactivation of GCN4, inactivation of the SPS sensing system resulted in a significant reduction of the ATO3 retrograde response but not its basal level of expression in \( \rho^- \) cells. Forsberg et al. (21) also categorized two classes of genes whose expression was SPS-dependent based on whether expression was stimulated by the addition of leucine to the medium. That study indicated that ATO3 was among the class of genes whose expression was unaffected by leucine. In our experiments, however, we detected an SPS-dependent leucine stimulation of ATO3 expression that was dependent on the integrity of the SPS sensor system. But that stimulation was only observed in \( \rho^- \) cells, possibly because, in the absence of leucine in the medium, the SPS-dependent expression of ATO3 in \( \rho^- \) cells was already at a maximal level. Further work will be required to resolve the differences between our results and those of Forsberg et al. (21) on the regulation of ATO3 expression. Finally, the possibility that amino acid sensing through the SPS system is a physiologically important aspect of regulation of ATO3 was suggested from the finding that, in cells defective in SPS sensing, ammonia production was decreased (22).

What is the relationship between the elevated expression of ATO3 (and other putative ammonia exporters, YCR010c and YNR002c; see Epstein et al., Ref. 2) in respiratory deficient \( \rho^- \) petite cells and ammonia production in respiratory competent cells in colonies that are in late stages of growth? There are a number of striking parallels between the overall physiological state of respiratory deficient cells and otherwise respiratory competent cells that are producing ammonia. In both cases, anaplerotic pathways such as the glyoxylate cycle and peroxisomal functions are induced so as to provide key metabolic intermediates such as acetyl-CoA and oxaloacetate to the mitochondrial tricarboxylic acid cycle (2, 14). These anaplerotic pathways are induced in respiratory deficient cells to ensure that sufficient amounts of \( \alpha \)-ketoglutarate and, in turn, glutamate and glutamine are available for biosynthetic reactions. Indeed, inactivation of the RTG genes in respiratory deficient cells or in cells with reduced mitochondrial function results in glutamate auxotrophy (4, 12). In the late stages of cell growth associated with ammonia production, Palková et al. (14) noted that the expression of a number of genes associated with mitochondrial respiratory function was down-regulated. Thus, metabolism in those cells appears to be reconfigured in much the same way as in respiratory deficient \( \rho^- \) cells, presumably to
accommodate those cells to reduced respiratory activity.

The data presented here underscore the notion that the retrograde response pathway links carbohydrate and nitrogen metabolism. Because of the limitation in α-ketoglutarate synthesis in respiratory deficient cells, there would likely be a stoichiometric imbalance between α-ketoglutarate and ammonia unless excess ammonia, which would be toxic to cells, was excreted. Moreover, genome-wide transcription analysis indicates that the expression of Gdh1p, which catalyzes the major route for glutamate synthesis from α-ketoglutarate and ammonia, is somewhat down-regulated in ρ0 cells (2). This would suggest that even less ammonia would be assimilated by respiratory deficient cells at the level of glutamate synthesis. The induction of ATO3 expression as shown here as well as the induction of other putative ammonium transporters in respiratory competent cells (14) could thus alleviate this potentially toxic ammonium imbalance by increased excretion of the excess ammonium. In respiratory competent cells, Gdh1p expression is regulated by a number of factors, including Hap4p, Leu3p, Gcn4p, and Gln3p (25), which reflect the role of glutamic dehydrogenase in coordinating carbohydrate and nitrogen utilization.

There are a number of parallels between the studies reported here and those of Moye-Rowley and co-workers (26, 27). They have shown that mitochondrial dysfunction, such as the loss of mitochondrial DNA or mutations in the inner mitochondrial membrane protein, Oxa1p, which is required for the assembly of cytochrome oxidase and the ATP synthase complex (28, 29), results in up-regulation of Pdr5p encoding a plasma membrane ABC transporter; when overproduced, the increased levels of Pdr5p result in pleiotropic drug resistance (PDR). Indeed, a genome-wide transcriptional analysis show that a large number of genes involved in pleiotropic drug resistance are up-regulated in ρ0 petites (30). The elevated expression of Pdr5p is due to increased expression of Pdr3p, a Cys6-Zn(II) transcription factor required for PDR expression. Like ATO3, this retrograde system shows only a modest (50–60%) dependence on the RTG genes. Also, in oxa1 cells the retrograde response appeared to be completely independent of the RTG genes. Although the signals that couple mitochondrial dysfunction to increased expression of PDR genes remains to be clarified, these and the current studies establish that the retrograde pathway is likely to involve multiple and not necessarily overlapping signals that trigger changes in nuclear gene expression in response to mitochondrial dysfunction.

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