HHS Public Access

Author manuscript

J Biol Chem. Author manuscript; available in PMC 2015 April 03.

Published in final edited form as:

J Biol Chem. 2003 September 19; 278(38): 36924–36933. doi:10.1074/jbc.M301829200.

Tor1/2 Regulation of Retrograde Gene Expression in *Saccharomyces cerevisiae* Derives Indirectly as a Consequence of Alterations in Ammonia Metabolism*

Jennifer J. Tate and **Terrance G. Cooper**‡

Department of Molecular Sciences, University of Tennessee, Memphis, Tennessee 38163

Abstract

Retrograde genes of *Saccharomyces cerevisiae* encode the enzymes needed to synthesize α-ketoglutarate, required for ammonia assimilation, when mitochondria are damaged or non-functional because of glucose fermentation. Therefore, it is not surprising that a close association exists between control of the retrograde regulon and expression of nitrogen catabolic genes. Expression of these latter genes is nitrogen catabolite repression (NCR)-sensitive, *i.e.* expression is low with good nitrogen sources (*e.g.* glutamine) and high when only poor (*e.g.* proline) or limiting nitrogen sources are available. It has been reported recently that both NCR-sensitive and retrograde gene expression is negatively regulated by glutamine and induced by treating cells with the Tor1/2 inhibitor, rapamycin. These conclusions predict that NCR-sensitive and retrograde gene expression should respond in parallel to nitrogen sources, ranging from those that highly repress NCR-sensitive transcription to those that elicit minimal NCR. Because this prediction did not accommodate earlier observations that *CIT2* (a retrograde gene) expression is higher in glutamine than proline containing medium, we investigated retrograde regulation further. We show that (i) retrograde gene expression correlates with intracellular ammonia and α-ketoglutarate generated by a nitrogen source rather than the severity of NCR it elicits, and (ii) in addition to its known regulation by NCR, NAD-glutamate dehydrogenase (*GDH2*) gene expression is down-regulated by ammonia under conditions where NCR is minimal. Therefore, intracellular ammonia plays a pivotal dual role, regulating the interface of nitrogen and carbon metabolism at the level of ammonia assimilation and production. Our results also indicate the effects of rapamycin treatment on *CIT2* transcription, and hence Tor1/2 regulation of retrograde gene expression occur indirectly as a consequence of alterations in ammonia and glutamate metabolism.

A primary interface of carbon and nitrogen metabolism in *Saccharomyces cerevisiae* occurs at the early reactions of the tricarboxylic acid cycle and retrograde genes (*CIT2*, *ACO1*, *IDH1*/*2*, and *DLD3*), encoding the enzymes that catalyze them (1–12). Expression of these genes, mediated by transcription factors Rtg1/3, is high in ammonia-grown yeast with damaged mitochondria and low when cells are grown in glucose-glutamate medium. These characteristics led to the conclusion that retrograde enzymes produce α-ketoglutarate,

---

*This work was supported by National Institutes of Health Grant GM-35642.

‡To whom correspondence should be addressed: Tel.: 901-448-6179; Fax: 901-448-8462; tcooper@utmem.edu.

needed for ammonia assimilation, in cells with damaged or inactive mitochondria. Earliest studies of the nutrient regulating retrograde gene expression identified glutamate as a negative regulator of the pathway (5). Subsequently, preferred nitrogen sources, glutamate and glutamine, were concluded to be negative regulators (7), and their presence was detected by the Ssy1 amino acid sensor (11). Most recently, Crespo *et al.* (12) concluded, from experiments using the metabolic inhibitor L-methionine sulfoximine, that glutamine negatively regulates expression of both retrograde and nitrogen catabolite repression (NCR)-sensitive genes (12).

NCR[1]-sensitive genes are those whose expression is high when only poor nitrogen sources (*e.g.* proline) are available and is low or repressed in the presence of good sources (*e.g.* glutamine, ammonia). Expression of these genes is mediated by GATA transcriptional activators, Gln3 and Gat1/Nil1 (13, 14). Gln3/Gat1 localize to the nuclei of cells grown under conditions where NCR-sensitive transcription is high (poor nitrogen source) and to the cytoplasm when it is low (good nitrogen source) (15, 16). Gln3 and Gat1 also form a complex with Ure2 that correlates with conditions that restrict GATA factors to the cytoplasm (17–20). Treating cells with rapamycin, a specific inhibitor of the phosphatidyl inositol-related kinases Tor1/2, results in dephosphorylation of Gln3, its nuclear localization, and thus increased NCR-sensitive transcription (17–20). The response of Gln3 phosphorylation and nuclear-cytoplasmic localization in rapamycin-treated wild type cells and mutants with defects in Tor1/2 signal transduction pathway components have led to the conclusion that NCR-sensitive transcription is regulated by this pathway.

Rapamycin also induces Rtg1/3-mediated retrograde transcription, and like Gln3, Rtg1/3 localizes to the nuclei of rapamycin-treated cells or cells where retrograde transcription is high and is cytoplasmic when such transcription is low (7, 12). Given the close physiological connection between ammonia assimilation and α-ketoglutarate synthesis, along with similarities observed in regulation of NCR-sensitive and retrograde transcription, it is easy to imagine the two regulons might be controlled together in parallel as reported.

Our interest in retrograde expression was stimulated by reports that Mks1 was a positive regulator of NCR-sensitive and retrograde gene expression (20, 21). The proposed regulatory pathways were as follows: NH_3 or excess nitrogen ⊣ Mks1p ⊣ Ure2p ⊣ Gln3p → *DAL5*, and rapamycin or limiting nitrogen ⊣ Torp → Tap42 ⊣ Mks1p → Rtg1/3p → *CIT2*, respectively. However, further investigation of these pathways led to the following conclusions. (i) Mks1p is a strong negative rather than positive regulator of *CIT2* expression (8, 9, 23). (ii) Mks1 does not affect NCR-sensitive expression of *DAL5* or *GAP1* except indirectly by altering the amount of α-ketoglutarate available for ammonia assimilation (23). (iii) In contrast with NCR-sensitive expression, retrograde expression does not correlate with the quality of the nitrogen source provided in the medium but does correlate the product of its catabolism, *i.e.* glutamate or ammonia (23). (iv) Mks1-mediated regulation of *CIT2* expression can be dissociated from the Tor signal transduction pathway, *i.e.* rapamycin

[1]The abbreviations used are: NCR, nitrogen catabolite repression; MSX, L-methionine sulfoximine; Arrows, positive regulation; Bars, negative regulation.

does not suppress Mks1p-mediated negative regulation of *CIT2* expression with proline as nitrogen source (23).

When data from Komeili *et al.* (7) and Crespo *et al.* (12), supporting the suggestion that NCR-sensitive and retrograde gene expression are regulated in common by glutamine, are compared with those of Tate *et al.* (23), several questions arise. (i) If glutamine is the nutrient regulating NCR-sensitive and retrograde transcription, why is *CIT2* expression greater with either glutamine or ammonia than with proline as nitrogen source even though the first two nitrogen sources support greater NCR than proline (23)? (ii) Why does rapamycin fail to induce retrograde gene expression with proline as sole nitrogen source? (iii) Is it possible that proline, not a commonly used nitrogen source in retrograde gene expression studies, possesses an unique and previously unrecognized property?

Investigating these questions, we discovered that retrograde (*CIT2*) gene expression closely correlates with the intracellular levels of ammonia and α-ketoglutarate generated by various nitrogen sources. Adding urea, a source of ammonia, to cells growing with proline as nitrogen source, deleting *URE2*, or adding rapamycin to ammonia grown cultures of yeast increases intracellular ammonia and *CIT2* expression in parallel. In addition to up-regulation of *CIT2* expression, ammonia also strongly down-regulates *GDH2* expression under conditions where NCR is minimal. Therefore, ammonia possesses a dual role, positively regulating expression of genes whose products synthesize α-ketoglutarate needed for its assimilation, and negatively regulating *GDH2*, whose product generates ammonia from glutamate. Finally, induction of retrograde gene expression by rapamycin, and hence its regulation by the Tor1/2 signal transduction pathway, can be accounted for as indirect consequences of alterations in nitrogen metabolism.

MATERIALS AND METHODS

Growth Conditions

S. cerevisiae strains (shown in Table I) were grown at 30 °C to mid-log phase ($A_{600\ nm}$ = 0.5–0.55) in Wickerham's minimal medium containing 2% glucose and the indicated nitrogen sources (proline, 0.2%; ammonia, glutamine, glutamate, asparagine, serine, or urea, 0.1%). Auxotrophic requirements were provided where necessary as follows: uracil (20 mg/liter), leucine (120 mg/liter), methionine (20 mg/liter), histidine, (20 mg/liter), lysine (40 mg/liter), arginine (20 mg/liter), tryptophan (20 mg/liter), and adenine (20 mg/liter). When a strain contained *rtg2* or *rtg3* mutations, glutamate (20 mg/liter) was included in the medium to cover the resulting auxotrophy. Wickerham's solid medium containing 2% glucose, 0.2% proline, 20 mg/liter uracil, 120 mg/liter leucine, and 20 mg/liter methionine was used to assess growth. Rapamycin was used at a final concentration of 200 or 300 ng/ml and was taken from a stock solution (1 mg/ml in 10% Tween 20 + 90% ethanol). Proline at 0.2 and 0.1% yields similar results; compare Fig. 1, *A*, *lane A versus B*, *lane A* and Fig. 3, *B*, *lane G versus lane A*. Ammonia at 0.1 and 0.5% yielded similar but not absolutely identical results. There was slightly higher *CIT2* expression at the higher ammonia concentration; compare Fig. 5*A*, *lane D versus* Fig. 1*B*, *lane C* and Fig. 3, *B*, *lane K versus lane E*.

Northern Blot Analyses

RNA preparation and Northern blot analyses were conducted as described earlier (24, 25). Primers used to synthesize radioactive DNA probes were as described earlier except for those listed below (23): *RTG2* (5′-AAATGCCCTATTCCCAGAGA-3′/5′-CAGGAGCAATCCTTTCGTTT-3′), *ACT1* (5′-TTCTTCCCAAGATCGA-AAATT-3′/5′-AACATACGCGCACAAAAGCA-3′), *DAL80* (5′-TGGAAA-AGTTTATGGTGAGCG-3′/5′-CCAGTTGCTTGCATTTTTCG-3′), and *GDH2* (5′-CCGACGATTTGATCGAACAA-3′/5′-AAAGGCTCCACTTT-TTCCAG-3′).

Transport Assays

Proline transport assays were performed as described earlier (26, 27). A 10-ml sample of each culture to be assayed was transferred to a pre-warmed 50-ml flask (to maintain a constant surface to volume ratio) containing ^{14}C uniformly labeled proline (final concentration, 4.3×10^{-6} M). 1.0-ml samples were transferred to a 0.45-μm filter and quickly washed eight times with 2.5 ml of cold, minimal proline medium. Filters were then transferred to aqueous scintillation fluid, and the radioactivity they contained was determined in a Beckman 5801 scintillation counter. Methylamine transport assays were performed similarly to those with proline, except that the 1-ml reaction mixture contained radioactive methylamine at a final concentration of 1.45×10^{-4} M.

Analysis of Intracellular Metabolite Concentrations

The procedures we used were modified from those of Dubois *et al.* (28). Cultures (470–480 ml) of yeast stains M970 and RR132 were grown overnight at 30 °C to an A_{600} of 0.50–0.55 in Wickerham's minimal medium, containing 2% glucose, uracil (20 mg/liter), lysine (40 mg/liter), histidine (20 mg/liter), arginine (20 mg/liter), and the indicated nitrogen source (ammonium sulfate, glutamic acid, urea, glutamine, or serine at a final a concentration of 0.1% or proline at 0.2%). Cells were collected on a Millipore filter (type HA; 0.45 μm), washed twice with 200 ml of ice-cold water, and placed in l0 ml of 0.3 M $HClO_4$ for 1 h at 0 °C (incubation for up to 4 h during reconstruction experiments using known concentrations of metabolites did not alter the results observed). Each extract was removed, and the filter and beaker were sequentially washed with 10 and 5 ml of ice-cold 0.3 M $HClO_4$, and the wash solutions were added to the extracts. Extracts were then neutralized to pH 7.2 with 1 M K_3P0_4, followed by centrifugation at 4 °C to remove cell debris and precipitated $KClO_4$. Supernatants were carefully collected, their volumes were determined (28–34 ml), and the concentrations of NH_4, α-ketoglutarate, and glutamate were measured. Supernatants were stored at −20 °C.

Ammonia Determination

Ammonia concentrations were measured using 17 ml of extract, with an ammonium-specific electrode (Accumet). Standard ammonia concentration curves (ammonium chloride 10^{-6} to 10^{-3} M) were prepared each time measurements were made. Reconstruction experiments, using known amounts of ammonium chloride and glutamine, verified the accuracy of our measurements and demonstrated that glutamine did not break down to ammonia and glutamate during extraction and ammonia assay procedures. Data are reported as nmol of

metabolite/mg dry weight of cells. Ammonia concentrations (12.8, 13.1, and 18.4 nmol/mg dry weight) obtained from three independent cell preparations provide an indication of the precision we observed in these assays. All metabolite assays were performed two or more times with similar or greater precision being observed in all cases.

α-Ketoglutarate

α-Ketoglutarate concentrations were determined using bovine liver Type II glutamate dehydrogenase (Sigma) and monitoring NADH disappearance at 340 nm. Reconstruction experiments, using known concentrations of α-ketoglutarate, verified the accuracy of the assay.

Glutamate Determination

Glutamate concentrations were determined using bovine liver Type II glutamate dehydrogenase (Sigma). Enzyme assay conditions were standard except for the use of 3-acetylpyridine-NAD in place of NAD. This was done, because we noticed limited breakdown of NADH when extended periods were required for the assay to reach completion. Reconstruction experiments verified the accuracy of the assays and demonstrated that glutamine did not contribute to the glutamate concentrations we observed.

RESULTS

The CIT2 Regulation Profile Is the Same with Glutamate and Proline as Nitrogen Source

Present experiments derive from two unexpected observations made during earlier studies of Mks1 and its participation in retrograde and NCR-sensitive gene expression (23): (i) *CIT2* expression is not detectable with proline as sole nitrogen source, and (ii) rapamycin is unable to induce *CIT2* expression with proline as nitrogen source even though such expression occurs at high levels in proline-grown *mks1*Δ cells (23). We investigated these observations further, because they heavily influence the interpretation of data concerning Tor1/2 regulation of retrograde transcription and the identity of metabolite(s) to which it potentially responds.

Retrograde (*CIT2*) gene expression is usually measured in cells provided with ammonia or glutamate as sole nitrogen source (1–5). In contrast, we previously assayed *CIT2* expression using proline as nitrogen source (23). Because *CIT2* expression, with or without rapamycin treatment, had not been measured previously with proline in other laboratories, our first objective was to determine whether the same results would be obtained if we used the more standard nitrogen source, glutamate. Failure to obtain the same results with proline and glutamate would argue that earlier observations with proline derived from some specific characteristic of proline or its metabolism. Therefore, we compared *CIT2* expression in proline- *versus* glutamate-grown cells (Fig. 1*A*). *CIT2* expression was similarly low with both proline and glutamate (*lanes A* and *B*). Deletion of *MKS1* dramatically increased *CIT2* expression with both nitrogen sources, demonstrating *CIT2* expression is also similarly down-regulated in minimal proline and glutamate media (*lanes C* and *D*).

The second observation, *i.e.* rapamycin does not induce *CIT2* expression in minimal proline medium, is important, because it cannot be easily rectified with the current model describing control of retrograde expression (7, 12, 20). According to that model, excess nutrients positively regulate Tor1/2, which in turn negatively regulates retrograde transcription via a regulatory pathway involving Tor1/2, Tap42, Rtg2, Mks1, and Rtg1/3. Because Tor1/2 is situated downstream of the nutrient signal, rapamycin treatment should inactivate Tor1/2 and induce retrograde transcription irrespective of the nitrogen source provided. Failure of rapamycin to induce *CIT2* expression in proline-grown cells suggests the nutrient signal enters the signal transduction pathway below rather than above Tor1/2. Again, it was important to know whether proline possessed some special characteristic, or is rapamycin-induced *CIT2* expression lost with glutamate as well? As shown in Fig. 1*B* (*lanes A*, *B*, *E*, and *F*), rapamycin is unable to induce *CIT2* expression with either proline or glutamate. Only with ammonia does rapamycin induce *CIT2* expression (Fig. 1*B*, *lanes C* and *D*). Therefore, the ability of rapamycin treatment to induce retrograde expression is clearly nitrogen source-dependent. The NCR-sensitive *DAL5* control experiment eliminates the possibility that rapamycin entry or action differs with ammonia *versus* glutamate as sole nitrogen source (Fig. 1*C*, *lanes C–F*). In sum, these data support the contention that both results obtained earlier with proline (23) likely derive from its degradation to glutamate.

Strain to Strain Variation in CIT2 Expression with Proline as Nitrogen Source

One of the serious challenges of comparing genome-wide or traditional transcription data from one laboratory to another are the effects of strain and growth condition differences, especially if they are unknown and hence cannot be taken into account. It is, for example, well known that ammonia elicits less NCR in S288C-derived strains than with those from a Σ1278b genetic background (29). This markedly alters expectations and interpretation of data when using ammonia as nitrogen source. Therefore, we determined whether retrograde, like NCR-sensitive, expression profiles exhibit strain-dependent differences. The epistasis experiment in Fig. 2*A* suggests this is the case. This experiment confirms the conclusion of Pierce *et al.* (10), *i.e. mks1* mutations are epistatic to those in *rtg2* and are the data upon which we based our support of it (23). In contrast with data in Fig. 1, high level *CIT2* expression occurs in strain 4852–1B (used by Pierce *et al.* (10)) growing in minimal proline medium (Fig. 2*A*, *lane A*). Expression with glutamate, however, is uniformly low in both figures. Additionally, *CIT2* expression with ammonia as nitrogen source and NCR-sensitive *DAL5* expression seen in Fig. 2 are the same as in Fig. 1. Therefore, data with glutamate and ammonia in Figs. 1 and 2 can be interpreted consistently in these two strains, whereas those obtained with proline cannot.

It was possible that data obtained with proline in Figs. 1 and 2 derive from major strain-dependent differences in the mechanism of *CIT2* regulation. We suspected, however, they more likely derived indirectly from variations in metabolism, thus altering the quantitative metabolic signals to which *CIT2* expression responds. To test this possibility, we compared growth and expression of *CIT2* and *DAL80* in three strains, M970, Σ1278b, and 4852–1B (the wild type used in Fig. 2). The former two strains are isogenic except for complementing auxotrophic markers (obtained by spontaneous mutation) in the diploid (26). In M970, *CIT2* is not expressed in the absence or presence of rapamycin with proline as sole nitrogen source

(Fig. 3*B*, *lanes A* and *B*). In Σ1278b, which grows slightly less well than M970 (Fig. 3*A*), *CIT2* is still not expressed in proline-grown cells but is detectably induced by rapamycin treatment (Fig. 3*B*, *lanes G* and *H*). In 4852–1B, which grew less well in minimal proline (and YPD) (data not shown) medium than either Sigma strain (Fig. 3*A*), *CIT2* is expressed at high levels regardless of whether rapamycin is present (Fig. 3*B*, *lanes M* and *N*). In other words, strain-dependent, growth-correlated differences in *CIT2* expression exist when cells are grown with proline as nitrogen source. In contrast with proline, *CIT2* expression in all three glutamate-grown cultures was undetectable (Fig. 3*B*, *lanes C*, *I*, and *O*) or minimal following rapamycin treatment (Fig. 3*B*, *lanes D*, *J*, and *P*). Finally, *CIT2* expression is uniformly high in all three rapamycin-treated strains grown in ammonia medium (Fig. 3*B*, *lanes F*, *L*, and *R*). However, in untreated ammonia-grown cultures, there was less *CIT2* expression in Sigma strains than 4852–1B (*lanes E*, *K*, and *Q*). Clearly, regulatory models based on results with strain 4852–1B would look quite different from those developed with data generated in Sigma-based strains unless strain differences are taken into account. To avoid the complications of strain differences, our experiments were performed in a single, well characterized genetic background, using strains that were as close to isogenic with Σ1278b as practical.

Are the Retrograde and NCR-sensitive Genes Regulated in Parallel by Glutamine?

A recent report concluded that glutamine regulates both retrograde and NCR-sensitive expression (12). This conclusion, based on studies with the inhibitor L-methionine sulfoximine, generates two testable predictions: (i) the two regulons might share glutamine-controlled transcription regulators, and (ii) gene expression for the two regulons should respond in parallel when assayed in cells provided with various nitrogen sources, taking into consideration that one regulon may possess greater glutamine sensitivity than the other. Therefore, we used Northern blot analysis to determine whether retrograde expression was influenced by loss of NCR-sensitive transcriptional activators, Gln3 and Gat1, or the negative regulator of their nuclear uptake, Ure2 (13, 14, 30). *DAL80* expression, which served as a positive control, was NCR-sensitive, rapamycin-responsive in wild type, and completely Gln3/Gat1-dependent (Fig. 4*A*). Similar data were found for other NCR-sensitive genes (data not shown). In contrast, *CIT2* expression profiles were similar in ammonia-grown wild type and *gln3*Δgat1Δ strains (Fig. 4*B*, *lanes A*, *B*, *E*, and *F*), arguing that *CIT2* expression is neither Gln3- nor Gat1-dependent. With glutamate as nitrogen source, there was almost no detectable *CIT2* expression in wild type cells regardless of whether they were treated with rapamycin. There was, however, detectable *CIT2* expression in untreated *gln3*Δgat1Δ cells, which increased upon rapamycin treatment (Fig. 4*B*, *lanes C*, *D*, *G*, and *H*). These data demonstrate the participation of Gln3/Gat1 and nitrogen metabolism in the response of *CIT2* expression to rapamycin treatment even though retrograde gene expression does not require these transcriptional activators.

Deletion of *URE2* derepresses NCR-sensitive *GAP1* expression (Fig. 4*D*). Also seen is the graded repression of *GAP1* expression that occurs with proline, glutamate, ammonia, and glutamine in wild type Sigma strains (Fig. 4*D*, *lanes A–D*). In contrast, deletion of *URE2* had no effect on the undetectable levels of *CIT2* expression observed in proline or glutamate medium (Fig. 4*C*, *lanes A*, *B*, *E*, and *F*). When corrected for the small difference in loading,

CIT2 expression with glutamine as nitrogen source was also similar in both strains (Fig. 4*C*, *lane D* and *H*). These data, along with those published earlier (15), suggest that downstream regulators of retrograde and NCR-sensitive transcription do not overlap in function. However, with ammonia as nitrogen source *CIT2* expression behaved exceptionally; deletion of *URE2* significantly increased *CIT2* expression (Fig. 4*C*, *lane G*). It is important to note that *GLN1* expression increases in a *ure2*Δ relative to wild type (Fig. 4*E*), and this increase in glutamine synthetase production should in turn increase glutamine production. According to the conclusions of Crespo *et al.* (12), deleting *URE2* should, if anything, diminish rather than increase *CIT2* expression in ammonia-grown cells.

The above experiments argue that if NCR-sensitive and retrograde transcription are regulated in parallel, it must be intracellular metabolite levels that control gene expression, because downstream transcription factors of the two sets of genes do not functionally overlap. This reasoning predicts that if retrograde and NCR-sensitive transcription are in fact regulated together, they should respond in parallel to nitrogen sources provided in the medium. Unfortunately, the predicted correlation is not observed experimentally. *CIT2* expression is not detectable with proline or glutamate as nitrogen source (Fig. 5*A*, *lanes A* and *B*), yet being poor and intermediate nitrogen sources, respectively, these amino acids support high and intermediate levels of NCR-sensitive *DAL80* expression (Fig. 5*B*, *lanes A* and *B*). It could be reasoned that *CIT2* expression is low with proline and glutamate as nitrogen source, because retrograde expression is more sensitive to glutamine levels than NCR-sensitive expression. Although it is possible for retrograde transcription to be more or less sensitive to glutamine levels than NCR-sensitive expression, it is not possible for it to be both more and less sensitive at the same time. Therefore, if *CIT2* expression is more sensitive to glutamine than NCR-sensitive expression, as required to explain *CIT2 versus DAL80* data with proline or glutamate, then it is difficult to understand why *CIT2* expression is so high when ammonia or glutamine, both of which repress *DAL80* expression, is provided (Fig. 5, *A*, *lanes C*, *D*, and *B*, *lanes C* and *D*).

CIT2 Expression Is Stimulated by Nitrogen Sources That Are Degraded to Ammonia

NCR-sensitive and retrograde transcription does not respond in parallel to growth with various nitrogen sources, as expected if both processes were regulated together (Fig. 5). However, *CIT2* expression does correlate with a nitrogen source's expected ability to produce ammonia, being high with ammonia or compounds degraded exclusively to ammonia and low with glutamate or nitrogen sources degraded to glutamate (Fig. 5*A*). Together these results raised the possibility that ammonia might positively regulate retrograde transcription. Urea supports highest *CIT2* expression, but data in Fig. 5 do not distinguish whether high *CIT2* expression occurs because urea elicits less NCR than glutamine, or because it is degraded to ammonia. These possibilities could be distinguished, however, if measurements were made under conditions where *CIT2* expression responds to ammonia production, but NCR does not decrease. The following identify such a condition. (i) Retrograde expression does not occur with proline, but does with urea (Fig. 5*A*, *lanes A* and *F*). (ii) Neither urea nor proline elicits significant NCR (Fig. 5*B*, *lanes A* and *F*), but they do generate glutamate and ammonia upon degradation. (iii) Simultaneously providing glutamate and ammonia as the nitrogen source represses NCR-sensitive expression more

than either compound alone (29). Therefore, adding urea to proline medium should increase *CIT2* expression if ammonia positively regulates retrograde transcription even though NCR either remains the same or potentially increases. Adding a very small amount of urea (0.025%) to proline-grown cells did not affect CIT2 expression, whereas a 2-fold larger amount (0.05%) increased it (Fig. 6*A*). *DAL80* expression was not affected in either case, arguing that urea additions had not changed the level of NCR (Fig. 6*B*). These are results expected of dose-dependent, positive regulation.

To further test the suggestion that ammonia positively regulates retrograde transcription, we followed the time-dependent response of *CIT2* expression after adding urea to proline-grown cells. *CIT2* expression was low at the outset and increased over 40-fold with time (Fig. 7*A*). Here, as before, *DAL80* expression did not change, demonstrating that NCR had not decreased (Fig. 7*B*). Cell doubling times prior to and after urea addition to the proline-grown cultures were identical within experimental error (Fig. 7*C*). These results are those expected if urea degradation to ammonia elicits *CIT2* expression. There is, however, an alternative interpretation of the data in Fig. 7, *i.e.* adding urea (a somewhat better nitrogen source than proline) excludes proline from the cell, thereby relieving proline/glutamate-dependent negative regulation of *CIT2* expression (31). This possibility is excluded by the observation that proline uptake prior to and following urea addition are identical (Fig. 7*D*). Similar results were observed when methylamine, a non-metabolized ammonia analogue, was added in place of urea. *CIT2* expression began to increase within 30 min of adding methylamine (data not shown). It must be emphasized, however, that results obtained with methylamine possess all of the caveats associated with the use of metabolic inhibitors.

Serine Stimulates CIT2 Expression while Repressing NCR-sensitive DAL80 Expression

As noted above, the fact that *CIT2* expression occurs with urea does not distinguish between urea being degraded to ammonia, which positively regulates *CIT2* expression, and *CIT2* expression occurring because urea elicits minimal NCR. The two possibilities can be distinguished, however, using a nitrogen source that is degraded to ammonia but, in contrast with urea, supports strong NCR, *i.e.* low *DAL80* expression. Serine, which is degraded by serine dehydratase (*CHA1*) to ammonia and pyruvate, is such a compound (32). *CIT2* but not *DAL80* is expressed when serine is provided as sole nitrogen source (Fig. 8*A*, *lane B*). Here, as in previous instances, *CIT2* expression correlates with the presence of ammonia rather than being regulated in parallel with NCR-sensitive gene expression.

CIT2 Expression Correlates with Intracellular Ammonia and α-Ketoglutarate Pools

Preceding *in vivo* experiments, using various nitrogen sources, are most consistent with the notion that *CIT2* expression correlates with a nitrogen source's ability to produce ammonia. We tested this correlation more directly by measuring metabolite pools in cells provided with the nitrogen sources in which *CIT2* expression was measured. Intracellular ammonia levels closely correlate with CIT2 expression, being lowest with glutamate and proline, roughly equal with ammonia, serine, and glutamine, and highest with urea as nitrogen source (Fig. 9*A*). A nearly perfect inverse correlation, with respect to ammonia, is observed for intracellular α-ketoglutarate, *i.e.* it is highest with glutamate or proline and low with ammonia or nitrogen sources degraded to it (Fig. 9*B*). Intracellular glutamate roughly

parallels α-ketoglutarate, but the -fold differences observed from one nitrogen source to another are much smaller (Fig. 9*C*).

Intracellular Ammonia Levels Explain the Response of CIT2 Transcription to Deletion of URE2 and Rapamycin Treatment

Rapamycin treatment or deletion of *URE2* markedly increases *CIT2* expression in ammonia-grown cells (Fig. 1*B*, *lanes C* and *D* and Fig. 4*C*, *lanes C* and *G*). Because increased *CIT2* expression occurred with ammonia but not glutamate or glutamine, we questioned whether it was a consequence of alterations in ammonia metabolism. Therefore, we assayed *MEP* gene (encoding the ammonia permeases) expression, ammonia transport (using the non-metabolized ammonia analogue, methylamine), and intracellular ammonia levels. *MEP2* expression increased dramatically when ammonia- or glutamine-grown cells were treated with rapamycin (Fig. 10*B*). There was also an increase with glutamate, but the effect was smaller (Fig. 10*B*, *lanes E* and *F*). Because rapamycin treatment similarly affected *MEP2* expression with both ammonia and glutamine, we assayed ammonia (methylamine) accumulation with glutamine- rather than ammonia-grown cells. This avoided the undesirable complication of cells being nitrogen-starved during the uptake assay. Such starvation occurs, because ammonia is a powerful inhibitor of methylamine uptake and hence cannot be present during the uptake assay. Rapamycin treatment of glutamine-grown cells increased methylamine uptake ~5-fold (Fig. 10*D*).

MEP2 gene expression increased in *ure2*Δ cells relative to wild type when glutamate, ammonia, or glutamine was provided as nitrogen source (Fig. 10*A*). However, the increase was greatest with ammonia. Methylamine accumulation was then compared in ammonia-grown wild type and *ure2*Δ strains. Here, the alternative format, involving nitrogen starvation, could not be avoided, because ammonia was the only nitrogen source with which the two strains differed. As with rapamycin treatment, methylamine transport was higher in the *ure2*Δ than wild type (Fig. 10*C*). The strength of the effect was smaller, because wild type accumulation values were higher than observed in Fig. 10*D*; this was the anticipated effect of nitrogen starvation. Even with the elevated wild type values, deletion of *URE2* increased methylamine uptake 2-fold.

Increased *MEP* gene expression and ammonia transport raised the possibility that ammonia levels increased in rapamycin-treated and *ure2*δ cells, which would account for increased *CIT2* expression. Therefore, we assayed ammonia, α-ketoglutarate, and glutamate levels under these conditions. Ammonia levels increased 3- and 5-fold, respectively, in the *ure2*Δ and rapamycin-treated cells growing in ammonia medium (Fig. 9, *D* and *E*). Significantly smaller changes occurred in α-ketoglutarate and glutamate levels. Moreover the changes were not consistent, *i.e.* α-ketoglutarate levels increased in the *ure2*Δ relative to wild type and decreased upon rapamycin treatment. Similarly small changes were observed for glutamate, as well, and as with α-ketoglutarate, the changes were not in a consistent direction.

Trans-acting Factors Required for Ammonia-induced CIT2 Expression

Four transcriptional regulatory proteins have been associated with retrograde transcription, Rtg2, Mks1, Rtg1, and Rtg3. To assess their participation in ammonia-induced *CIT2* expression or lack of it with proline, we compared steady state *CIT2* mRNA levels in wild type and mutants with defects in these retrograde regulatory proteins. Rtg2 is required for ammonia-induced *CIT2* expression (Fig. 2, *lane I*) as is Rtg3 (Fig. 5*C*).

Ammonia Is a Strong Negative Regulator of GDH2 Expression

When glutamate or proline is provided as sole nitrogen source, it must be degraded to ammonia so that glutamine and other nitrogenous compounds can be synthesized. However, if the ammonia were to be re-assimilated into glutamate, a futile cycle would ensue. This prompted the question, why isn't *CIT2* expressed in response to glutamate-derived ammonia? One explanation posits that expression of *GDH2* (encoding NAD-glutamate dehydrogenase responsible for degrading glutamate to ammonia) is NCR-sensitive (33). This explanation, however, does not account for the *GDH2* expression profile in Fig. 8*C*, because urea is not a highly repressive nitrogen source as indicated by *DAL80* expression (Fig. 8*D*, *lane C*). Yet *GDH2* expression is undetectable with urea (Fig. 8*C*, *lane C*). This experiment suggests a response to ammonia, which drastically down-regulates *GDH2* expression beyond what occurs with NCR.

DISCUSSION

Ammonia Is a Pivotal Regulator of Ammonia Assimilation and Production

Four conclusions derive from the above experiments. (i) Intracellular ammonia positively regulates retrograde transcription. (ii) Ammonia negatively regulates *GDH2* expression under conditions where transcription of even highly NCR-sensitive genes is not diminished. (iii) Apparent Tor1/2 regulation of retrograde gene expression is nitrogen source-dependent and an indirect consequence of alterations that occur in nitrogen metabolism. (iv) The response of retrograde gene expression to rapamycin treatment and growth with various nitrogen sources is strain-dependent.

This work identifies a pivotal dual regulatory role for ammonia in the integration of carbon and nitrogen metabolism (Fig. 11). In its first role, ammonia, directly or indirectly through α-ketoglutarate, stimulates retrograde gene expression and thus the enzymes that synthesize α-ketoglutarate, which are required for ammonia assimilation under fermentative growth or other conditions where the tricarboxylic acid cycle is inoperative. Ammonia and/or α-ketoglutarate was chosen as the metabolite to which retrograde transcription most likely responds based on the following reasoning. *CIT2* expression varies over a 10- to 20-fold range as a function of nitrogen source and genetic composition of the strain. Correlating with this change, intracellular ammonia and α-ketoglutarate both vary ~14-fold under these conditions. Ammonia varies in parallel with *CIT2* expression, whereas α-ketoglutarate levels vary just oppositely, being lowest when ammonia and *CIT2* expression are greatest. The inverse relationship between ammonia and α-ketoglutarate levels is not surprising, because α-ketoglutarate is stoichiometrically consumed, becoming the carbon backbone of glutamate, as ammonia is assimilated. In contrast, when glutamate or compounds degraded

to it are utilized, α-ketoglutarate is a stoichiometric by-product of ammonia production and in the absence of a functional tricarboxylic acid cycle can be expected to accumulate to higher levels as observed. Because the levels of both ammonia and α-ketoglutarate strongly correlate with *CIT2* expression, it is not possible with present data to unequivocally choose one over the other as the regulatory metabolite most proximal to transcription. However, it is ammonia levels that are most directly influenced by the nitrogen source provided. Changes in ammonia levels in turn alter α-ketoglutarate as a consequence of assimilation. Data with the *ure2*Δ and rapamycin-treated cells provided with ammonia as nitrogen source favor ammonia, because changes in α-ketoglutarate are small, but they alone are not sufficient to settle the issue.

We cannot categorically exclude glutamate as a potential regulator of retrograde transcription, but do not favor it for several reasons. (i) Glutamate varies much less than either ammonia or α-ketoglutarate, *i.e.* under 3-fold as a function of nitrogen source. There is, however, a potential caveat associated with this observation, *i.e.* amino acids do not partition equally between the cytoplasm and vacuole. (ii) Glutamate, like α-ketoglutarate, varies less than 2-fold in the *ure2*Δ and rapamycin-treated cells and does so inconsistently, *i.e.* it decreases in the *ure2*Δ and increases upon rapamycin treatment. (iii) Glutamate levels are about the same in ammonia-grown and urea-grown cells, yet *CIT2* expression is much higher with the latter than the former nitrogen source. (iv) Intracellular glutamate levels can be altered in multiple ways that have little to do with ammonia or α-ketoglutarate if ammonia or compounds degraded to it is not present as a nitrogen source. Each of these perturbations would also alter retrograde gene expression if glutamate was the molecule to which it responds. However, it is only when ammonia is present that retrograde gene expression is advantageous to the cell. In fact, it is probably counterproductive under most other circumstances. Glutamine is not favored as a negative regulator, because there is more glutamine in glutamine-grown than proline-grown yeast cells (34), yet *CIT2* expression is much higher with the former than the latter nitrogen source.

Ammonia is depicted in Fig. 11 as a positive regulator of Rtg2, based on the observation that Rtg2 is required for ammonia-stimulated *CIT2* expression and that *CIT2* expression is constitutive in a *mks1*Δ. However, no data eliminate the possibility of an intermediate molecule(s) functioning between ammonia (or α-ketoglutarate) and the retrograde regulatory proteins. In addition, a model in which α-ketoglutarate functions as a positive regulator of Mks1 or a negative regulator of Rtg2 is equally easy to envision.

The second role of ammonia in the integration of carbon and nitrogen metabolism occurs when glutamate or compounds degraded to it are being utilized. Under these conditions, cells must produce sufficient ammonia to meet the cell's biosynthetic needs for glutamine and other nitrogenous compounds, but no more, because at high concentration, ammonia is toxic and could also result in an ammonia-glutamate futile cycle. Such overproduction is avoided through regulation of NAD-glutamate dehydrogenase (encoded by *GDH2*). One mode of regulation is the NCR sensitivity of *GDH2* expression (33). In addition, this work identifies a second level of regulation that appears to act in the absence of demonstrable NCR, *e.g.* with urea as nitrogen source.

The Tor1/2 Signal Transduction Pathway Influences Retrograde Gene Expression Indirectly as a Result of Its Effects on Nitrogen Metabolism

The current model describing Tor1/2 regulation of retrograde transcription cannot easily accommodate the nitrogen source and nitrogen metabolism dependence of rapamycin-induced *CIT2* expression. In bare outline, this model posits glutamine → Tor1/2 ⊣ Rtg3 → *CIT2*. Inhibition of the pathway by rapamycin can be similarly represented as follows: rapamycin ⊣ Tor1/2 ⊣ Rtg3 → *CIT2* (12). Nutrients and rapamycin both act on Tor1/2 but generate opposite results, *i.e.* glutamine positively regulates Tor1/2, and rapamycin inhibits them. If rapamycin inhibits the Tor1/2 kinases, the nitrogen source upon which the cells are growing should not influence retrograde transcription in rapamycin-treated cells. Yet rapamycin-induced *CIT2* expression occurs with ammonia but not proline or glutamate as nitrogen source. These are results expected if the nitrogen source-dependent signal enters the pathway regulating retrograde transcription downstream of Tor1/2, which would occur if that regulation derived indirectly as a consequence of Tor1/2 influence on nitrogen metabolism. This interpretation is further supported by the following facts. (i) *CIT2* expression can be induced in glutamate-grown cells if *GLN3* and *GAT1* are deleted even though these transcription factors are not required for retrograde transcription (Fig. 4*A*). (ii) Rapamycin-induced *CIT2* expression in ammonia-grown cells can be explained by increased *MEP* gene expression, ammonia transport, and intracellular accumulation following rapamycin treatment.

The most difficult data to explain derive from studies with glutamine synthetase inhibitor, L-methionine sulfoximine (MSX), which causes glutamine depletion, nuclear localization of TOR-inhibited transcription factors Gln3, Rtg1, and Rtg3, and increased retrograde and NCR-sensitive gene expression (12). Although MSX inhibits glutamine synthetase, it is not enzyme-specific. MSX is also a known substrate or inhibitor of γ-glutamylcysteine synthetase, L-amino acid oxidase, glutamine transaminase, and γ-cystathionase (22, 35, 36). Structurally, MSX is perhaps most accurately viewed as a glutamate analogue that likely inhibits, to varying degrees, all enzymatic reactions for which glutamate is a substrate. That more than the intended inhibition of glutamine synthetase, and hence glutamate incorporation into glutamine, is occurring in MSX-treated cells is perhaps indicated by the observation that MSX treatment decreases intracellular glutamine by 13 μmol/g of protein compared with a 3-fold greater 42 μmol/g of protein increase in glutamate. Until all of the effects of MSX inhibition have been identified and evaluated with respect to their direct and indirect influences on ammonia and glutamate metabolism, it will be difficult to rigorously explain the MSX results.

Strain Variation Is an Important Variable in the Interpretation of Retrograde Expression Data

This work demonstrates that strain variation can markedly influence the data observed for retrograde gene expression just as it does for NCR-sensitive gene expression. In fact, data in Fig. 4*A* suggest the difference between strains that do and do not exhibit rapamycin-induced *CIT2* expression in glutamate medium likely resides in a gene whose expression requires Gln3 and/or Gat1. Given such variations, there will always be some risk involved in extrapolating data from one strain to another unless there is an independent way of detecting

these variations and appropriately accounting for them. On the other hand, such variations, when understood, represent additional tools for elucidating how these complex regulatory systems communicate with one another.

Acknowledgments

We thank Drs. Reed Wickner and Ted Powers for very generously providing strains, Tim Higgins for preparing the artwork, and the University of Tennessee Yeast Group for suggestions to improve the manuscript.

References

1. Liao XS, Small WC, Srere PA, Butow RA. Mol Cell Biol. 1991; 11:38–46. [PubMed: 1986232]
2. Liao X, Butow RA. Cell. 1993; 72:61–71. [PubMed: 8422683]
3. Chelstowska A, Butow RA. J Biol Chem. 1995; 270:18141–18146. [PubMed: 7629125]
4. Velot C, Haviernik P, Lauquin GJ. Genetics. 1996; 144:893–903. [PubMed: 8913736]
5. Zhegchang L, Butow RA. Mol Cell Biol. 1999; 19:6720–6728. [PubMed: 10490611]
6. Sekito T, Thornton J, Butow RA. Mol Biol Cell. 2000; 11:2103–2115. [PubMed: 10848632]
7. Komeili A, Wedaman KP, O'Shea EK, Powers T. J Cell Biol. 2000; 151:863–878. [PubMed: 11076970]
8. Sekito T, Liu Z, Thornton J, Butow RA. Mol Biol Cell. 2002; 13:795–804. [PubMed: 11907262]
9. Dilova I, Chen CY, Powers T. Curr Biol. 2002; 12:389–395. [PubMed: 11882290]
10. Pierce MM, Maddelein ML, Roberts BT, Wickner RB. Proc Natl Acad Sci U S A. 2001:13213–13218. [PubMed: 11687605]
11. Liu Z, Sekito T, Epstein CB, Butow RA. EMBO J. 2001; 20:7209–7219. [PubMed: 11742997]
12. Crespo JL, Powers T, Fowler B, Hall MN. Proc Natl Acad Sci U S A. 2002; 99:6784–6789. [PubMed: 11997479]
13. Hoffman-Bang J. Mol Biotechnol. 1999; 12:35–73. [PubMed: 10554772]
14. Cooper TG. FEMS Microbiol Rev. 2002; 26:223–238. [PubMed: 12165425]
15. Cox KH, Rai R, Distler M, Daugherty JR, Coffman JA, Cooper TG. J Biol Chem. 2000; 275:17611–17618. [PubMed: 10748041]
16. Cox KH, Tate JJ, Cooper TG. J Biol Chem. 2002; 277:37559–37566. [PubMed: 12140287]
17. Beck T, Hall MN. Nature. 1999; 402:689–692. [PubMed: 10604478]
18. Cardenas ME, Cutler NS, Lorenz MC, Di Como CJ, Heitman J. Genes Dev. 1999; 13:3271–3279. [PubMed: 10617575]
19. Bertram PG, Choi JH, Carvalho J, Ai W, Zeng C, Chan TF, Zheng XFS. J Biol Chem. 2000; 275:35727–35733. [PubMed: 10940301]
20. Shamji AF, Kuruvilla FG, Schreiber SL. Curr Biol. 2000; 10:1574–1581. [PubMed: 11137008]
21. Edskes HK, Hanover JA, Wickner RB. Genetics. 1999; 153:585–594. [PubMed: 10511541]
22. Cooper AJ, Stephani RA, Meister A. J Biol Chem. 1976; 251:6674–6682. [PubMed: 977592]
23. Tate JJ, Cox KH, Rai R, Cooper TG. J Biol Chem. 2002; 277:20477–20482. [PubMed: 11923302]
24. Rai R, Tate JJ, Cooper TG. J Biol Chem. 2003; 278:12826–12833. [PubMed: 12562760]
25. Cox KH, Pinchak AB, Cooper TG. Yeast. 1999; 15:703–713. [PubMed: 10392447]
26. Turoscy V, Cooper TG. J Bacteriol. 1982; 151:1237–1246. [PubMed: 7050082]
27. Sumrada RA, Cooper TG. J Bacteriol. 1976; 125:1048–1056. [PubMed: 3491]
28. Dubois E, Grenson M, Wiame JM. Eur J Biochem. 1974; 48:603–616. [PubMed: 4614980]
29. Bossinger J, Cooper T. Biochem Biophys Res Commun. 1975; 66:889–892. [PubMed: 241348]
30. Crespo JL, Hall MN. Microbiol Mol Biol Rev. 2002; 66:579–591. [PubMed: 12456783]
31. Watson TG. J Gen Microbiol. 1977; 103:123–126.
32. Petersen JG, Kielland-Brandt MC, Nilsson-Tillgren T, Bornaes C, Holmberg S. Genetics. 1988; 119:527–534. [PubMed: 2841185]

33. Miller SM, Magasanik B. Mol Cell Biol. 1991; 11:6229–6247. [PubMed: 1682801]
34. Watson TG. J Gen Microbiol. 1976; 96:263–268. [PubMed: 792385]
35. Griffith OW, Meister A. J Biol Chem. 1978; 253:2333–2338. [PubMed: 24637]
36. Richman PG, Orlowski M, Meister A. J Biol Chem. 1973; 248:6684–6690. [PubMed: 4147652]

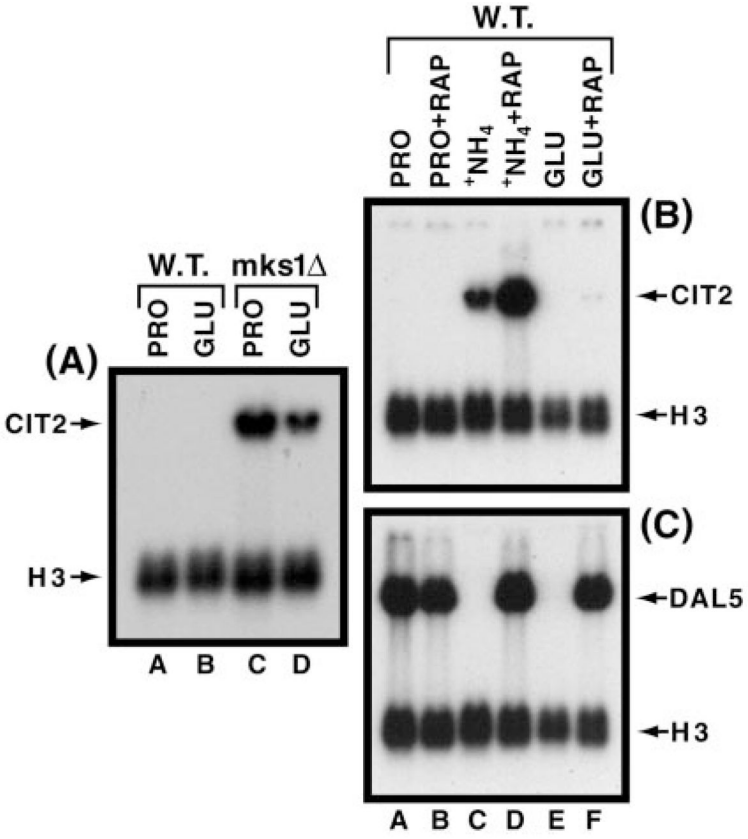

Fig. 1. Northern blot analyses of the effects generated by rapamycin treatment or deleting *MKS1* on retrograde (*CIT2*) and NCR-sensitive (*DAL5*) expression in *S. cerevisiae* cells provided with proline (*PRO*), glutamate (*GLU*), or ammonia ($^{+}NH_4$) as sole nitrogen source

The *S. cerevisiae* strains used were M970 (*W.T.*) and YHE677 (*mks1*Δ). Rapamycin (300 ng/ml) was added for 30 min where indicated (+*RAP*). Histone H3 expression was used as the loading and transfer standard.

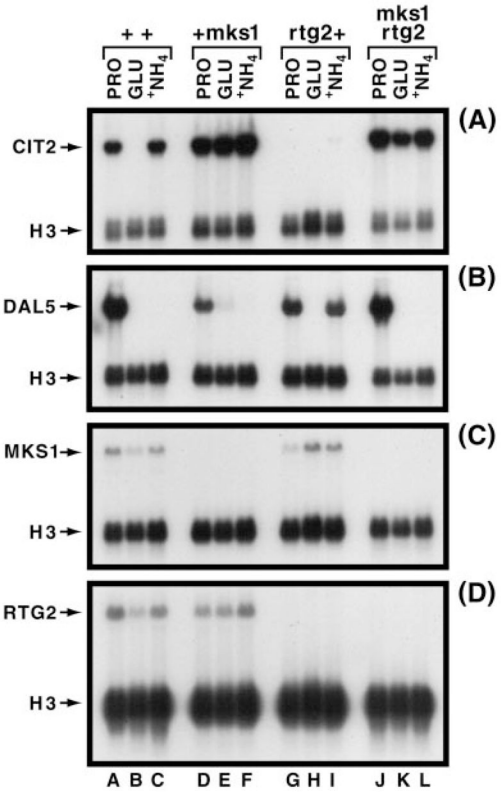

Fig. 2. Epistasis relationship of *mks1*Δ and *rtg2*Δ mutations on retrograde (*CIT2*) and NCR-sensitive (*DAL5*) expression determined by Northern blot analyses

The *S. cerevisiae* strains used were 4852–1B (++), 4852–1D (+*mks1*), 4852–1C (*rtg2*+), and 4852–1A (*mks1 rtg2*). Steady state *MKS1* and *RTG2* mRNA were monitored by Northern blot analyses to verify the mutant phenotypes.

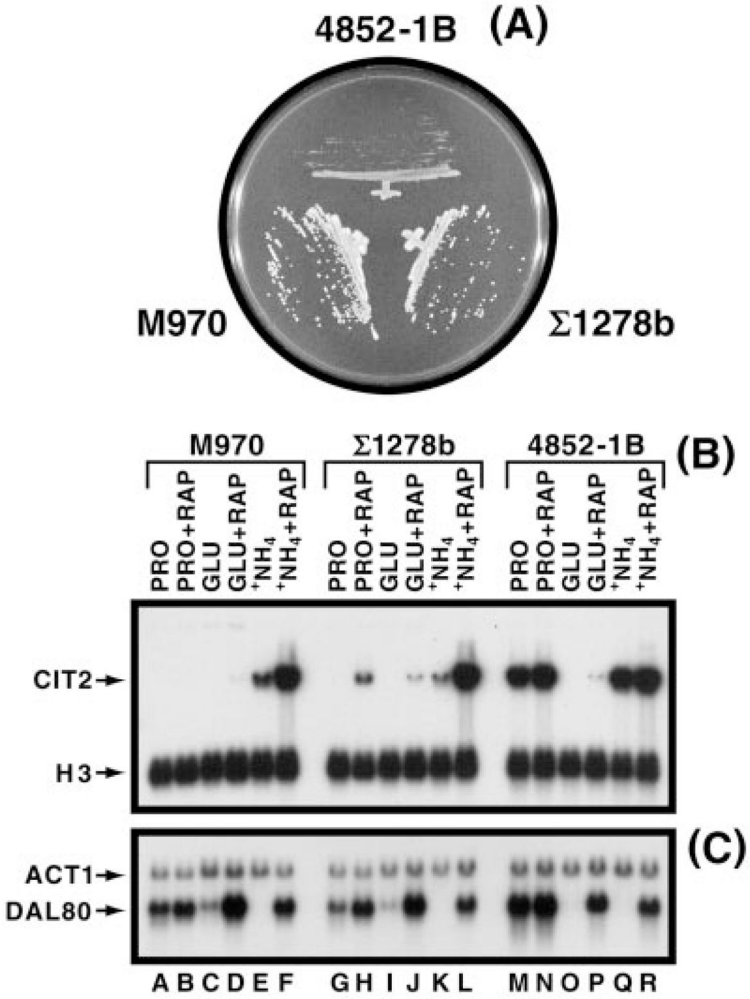

Fig. 3. Growth correlated strain differences in retrograde gene expression

Panel A, growth of strains M970, 4852-1B, and Σ1278b in minimal proline medium. *Panels B* and *C*, Northern blot analyses of *CIT2* and *DAL80* expression in minimal proline, glutamate, and ammonia medium in the presence (+*RAP*) or absence of rapamycin (300 ng/ml). *ACT1* was used as the loading standard for *DAL80*, because *H3* and *DAL80* migrate too close to one another in the electrophoretic gel. The growth difference between strains M970 and Σ1278b is more clear if the plate is viewed after a shorter time. However, at that time strain 4852–1B had not yet grown to a point where it could be appropriately photographed.

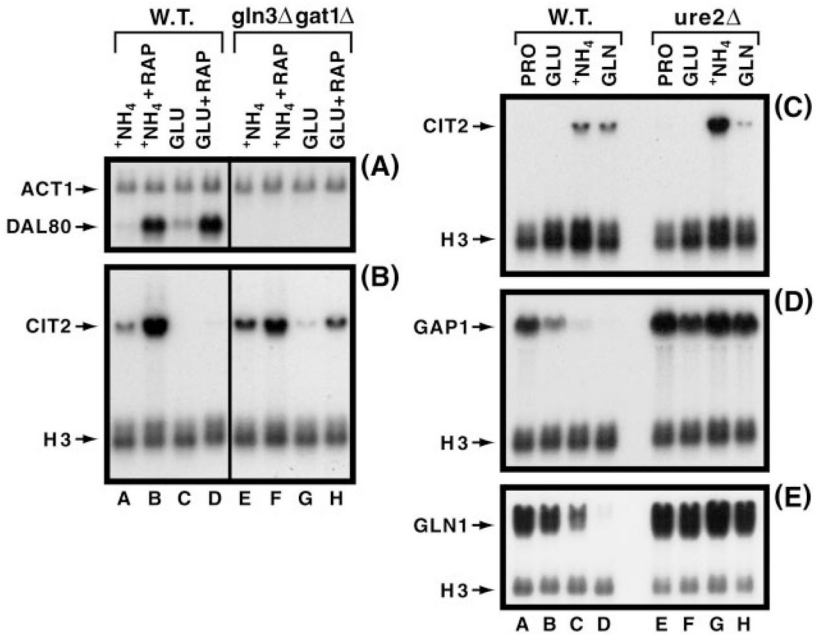

Fig. 4.

Northern blot analysis of retrograde (*CIT2*) and NCR-sensitive (*DAL80*) expression in wild type (*W.T.*; TCY1) and *gln3*Δ*gat1*Δ (YKHC7) strains provided with ammonia ($^{+}NH_4$) or glutamate (*GLU*) in the presence or absence of rapamycin (300 ng/ml) or wild type (M970) and *ure2*Δ (RR132) strains provided with proline (*PRO*), glutamate (*GLU*), ammonia ($^{+}NH_4$), or glutamine (*GLN*) as sole nitrogen source.

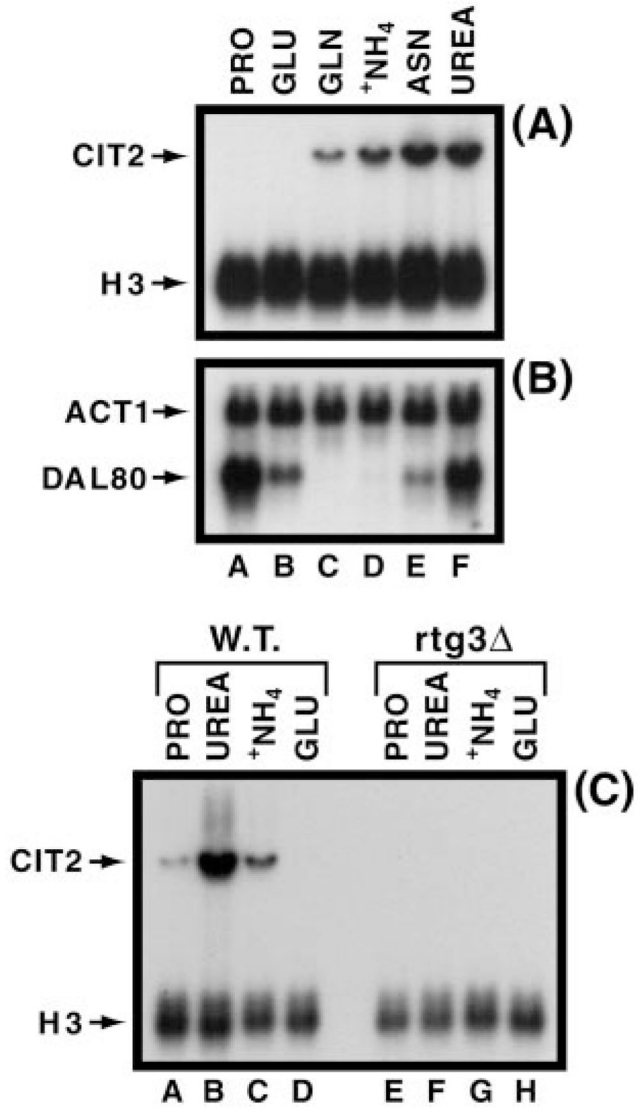

Fig. 5.

Panels A and *B*, Northern blot analyses of retrograde (*CIT2*) and NCR-sensitive (*DAL80*) expression in M970 provided with nitrogen sources degraded to glutamate (proline, *PRO*; glutamate, *GLU*; glutamine, *GLN*) or ammonia ($^{+}NH_4$; asparagine, *ASN*; urea). *Panel C*, Northern blot analysis of *CIT2* expression in wild type (K699) and *rtg3*Δ (EYO735) strains grown in minimal medium with the indicated nitrogen sources, proline (*PRO*), urea, ammonia ($^{+}NH_4$), or glutamate (*GLU*).

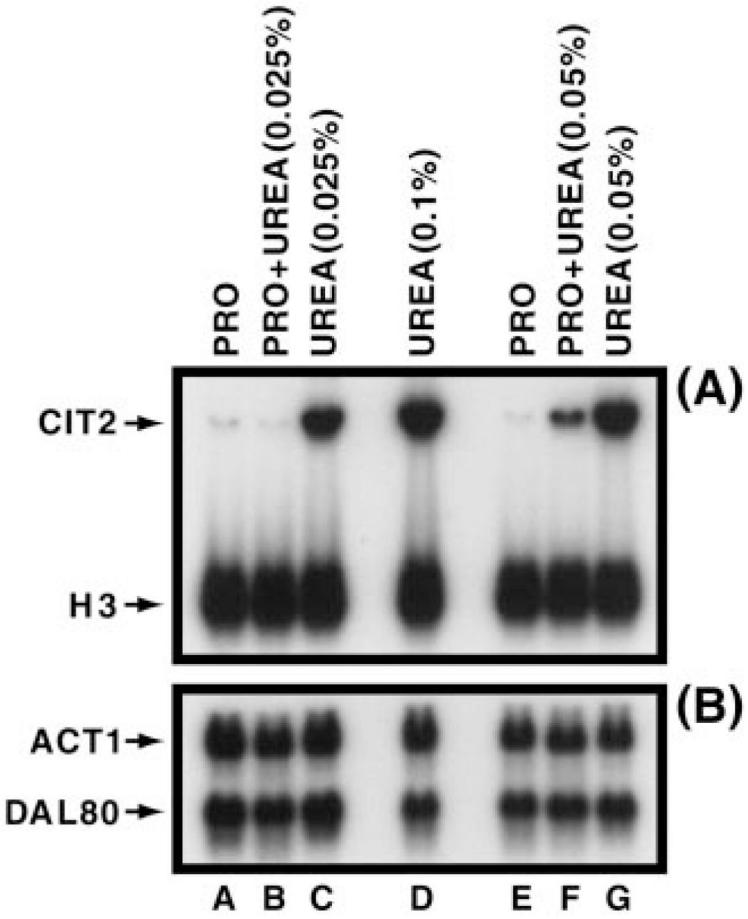

Fig. 6. The effect of urea on retrograde (*CIT2*) expression in cultures of M970 grown in proline (*PRO*) (*lanes A* and *E*), proline + urea (0.025 or 0.05%; *lanes B* and *F*, respectively), or urea (0.025, 0.05, or 0.1%; *lanes C*, *D*, and *G*, respectively)

DAL80 expression was used to assess NCR-sensitive transcription, and *ACT1* expression was used to assess loading and transfer variations.

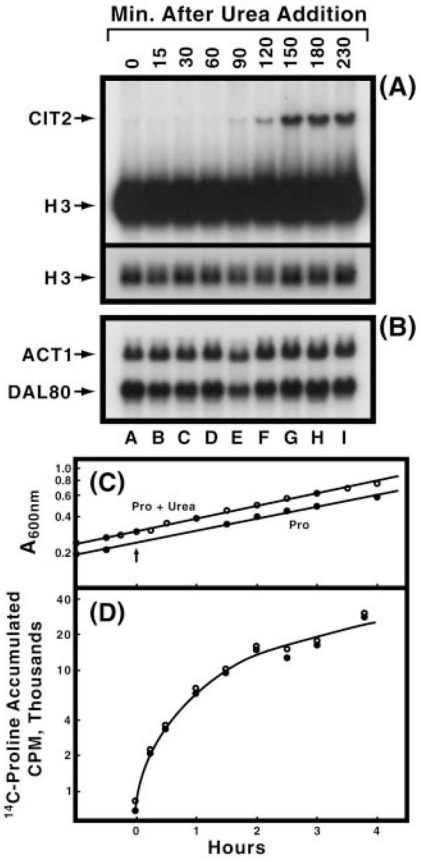

Fig. 7. Time course of *CIT2* expression following addition of urea to cells growing in minimal proline medium

A wild type (M970) culture was grown to an $A_{600\ nm}$ of 0.3. A sample was taken (*zero point*), and urea was added to achieve a final concentration of 0.1%. Samples were taken thereafter at the times indicated for Northern blot-based RNA analyses (*panels A* and *B*) and absorbance determinations (*panel C*). Short and long exposures of the H3 hybrids are shown in *panel A*. ^{14}C-proline accumulation was measured (*panel D*) as described under "Materials and Methods."

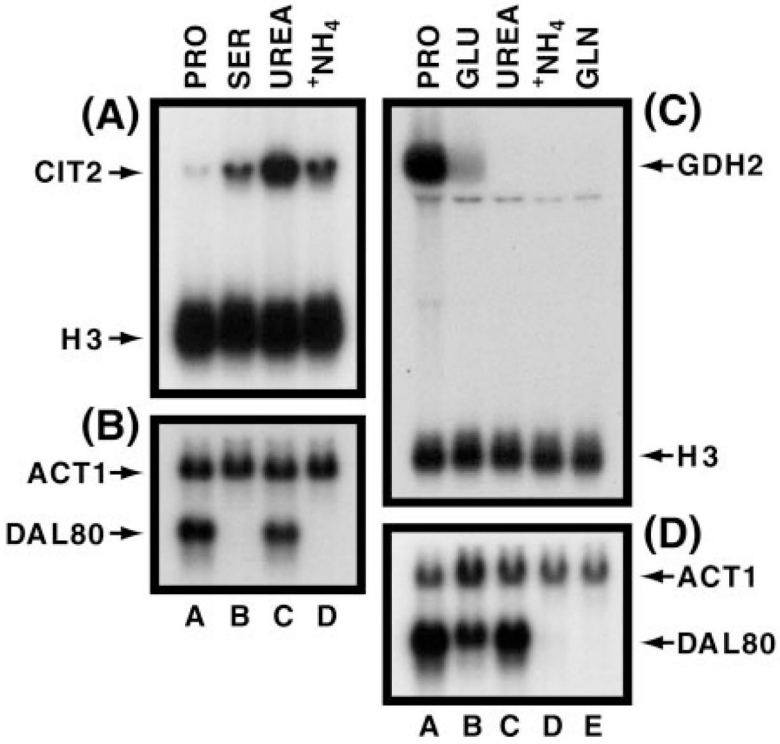

Fig. 8.

Northern blot analyses of *CIT2*, *GDH2*, and *DAL80* expression in wild type strains (Σ1278b, *panels A* and *B*; M970, *panels C* and *D*) cultured with proline (*PRO*), serine (*SER*), glutamate (*GLU*), glutamine (*GLN*), urea, or ammonia ($^{+}NH_4$) as sole nitrogen source.

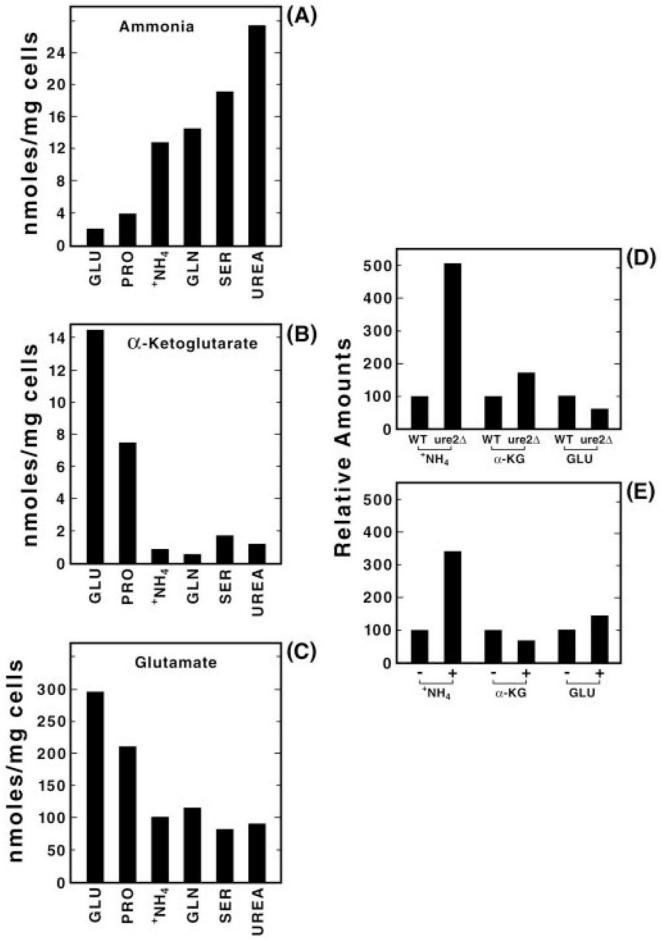

Fig. 9.

Panels A–C, intracellular pools of ammonia, α-ketoglutarate, and glutamate in wild type (M970) cells provided with proline (*PRO*), serine (*SER*), glutamate (*GLU*), glutamine (*GLN*), urea, or ammonia ($^{+}NH_4$) as sole nitrogen source. *Panel D* and *E*, relative amounts of ammonia, α-ketoglutarate, and glutamate in wild type (M970) and *ure2*Δ (RR132) strains provided with ammonia as sole nitrogen source. Where indicated (+ *RAP*), cultures were incubated for 30 min with 200 ng/ml rapamycin. Concentrations of ammonia, α-ketoglutarate, and glutamate were set as 100%, and concentrations of the remaining samples are expressed relative to these values.

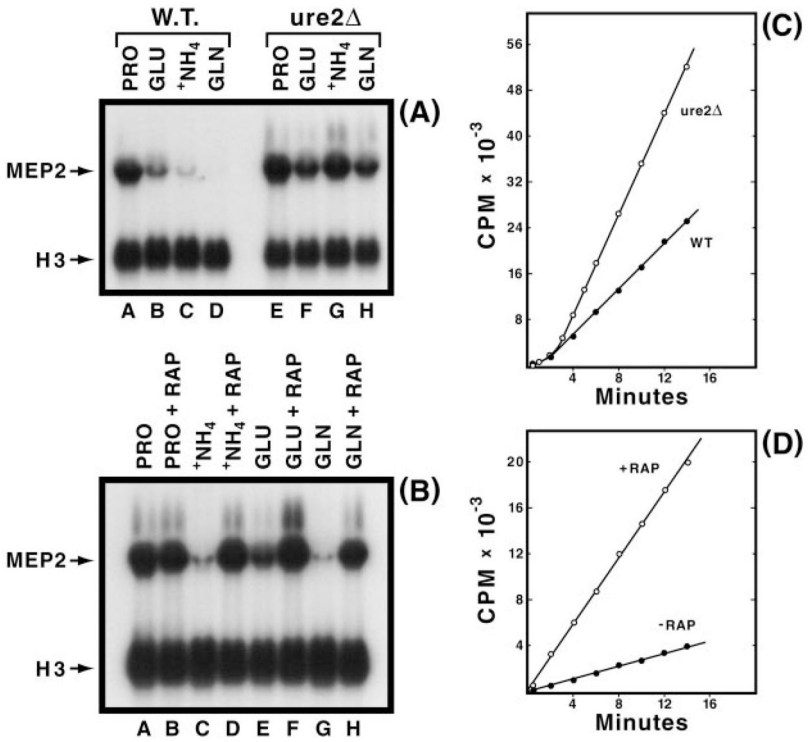

Fig. 10.

Panels A and B, MEP2 gene expression in wild type (M970) and *ure2*Δ (RR132) cultures provided with the nitrogen source. Where indicated (+*RAP*), cultures were incubated 30 min with 300 ng/ml rapamycin. *Panel C*, methylamine accumulation in ammonia-grown wild type (M970) and *ure2*Δ (RR132) growing in minimal ammonia medium. Following growth to early log phase, 10 ml of cells were harvested by filtration, washed three times with 12 ml of nitrogen-free minimal medium, and transferred to a flask containing radioactive methylamine. Methylamine accumulation was then assayed as described under “Materials and Methods.” *Panel D*, methylamine accumulation in glutamine-grown wild type cells (M970) cells. Where indicated (+*RAP*), cultures were incubated 30 min with 200 ng/ml rapamycin.

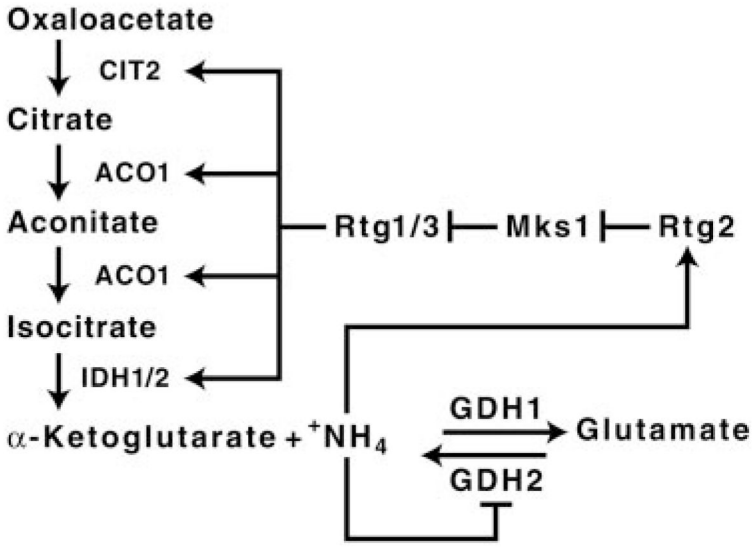

Fig. 11.

Working model summarizing the pivotal, dual role of ammonia as a positive regulator of retrograde gene expression and negative regulator of *GDH2* expression.

Table I

S. cerevisiae strains used in this work

Strain number	Genotype
M970	*MATa, lys5/MATα, lys2*
YHE677	*MATa/MATα, mks1Δ::G418/mks1Δ::G418, ura3/+*
4852–1A	*MATα, ura2, leu2, met15, mks1::G418, rtg2::G418*
4852–1B	*MATα, ura2, leu2, met15*
4852–1C	*MATα, ura2, rtg2::G418*
4852–1D	*MATα, ura2, mks1::G418*
Σ1278b	*MATα*
TCY1	*MATα, lys2, ura3*
YKHC7	*MATα, lys2, ura3, gat1Δ::hisG, gln3Δ::KANMX4*
RR132	*MATα, lys2, ura3, trp1, ure2::TRP1/MATα, lys2, ura3, trp1, ure2::TRP1*
K699	*MATα, ade2–1, trp1–1, can1–100, leu2–3,112, his3–11, 15, ura3, GAL+*
EY0735	*MATα, ade2–1, trp1–1, can1–100, leu2–3,112, his3–11,15, ura3, rtg3Δ::TRP1, GAL+*