Rtg3p, a Basic Helix-Loop-Helix/Leucine Zipper Protein that Functions in Mitochondrial-induced Changes in Gene Expression, Contains Independent Activation Domains*

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Rtg3p and Rtg1p are basic helix-loop-helix/leucine zipper protein transcription factors in yeast that interact and bind to sites in an upstream activation sequence element in the 5’-flanking region of CIT2, a gene encoding a peroxisomal isoform of citrate synthase. These factors are required both for basal expression of CIT2 and its elevated expression in cells with dysfunctional mitochondria, such as in respiratory-deficient petite cells lacking mitochondrial DNA (p°). This elevated expression of CIT2 is called the retrograde response. Here we show that fusion constructs between the Gal4p DNA binding domain and Rtg3p transactivate the expression of a LacZ reporter gene under the control of a GAL1 promoter element. We have identified two activation domains in Rtg3p: a strong carboxyl-terminal domain from amino acids 375–486, and a weaker amino-terminal domain from amino acids 1–175; neither of these activation domains contain the bHLH/Zip motif. We have also identified a serine/threonine-rich domain of Rtg3p within amino acids 176–282 that is inhibitory to transactivation. In addition, the transcriptional activity of the Gal4-Rtg3p fusion proteins does not require either Rtg1p or Rtg2p; the latter is a protein containing an hsp70-like ATP binding domain that is also necessary for CIT2 expression. In contrast, transcriptional activation by Gal4-Rtg1p fusion proteins requires the Rtg1p basic helix-loop-helix/leucine zipper protein domain, as well as Rtg3p and Rtg2p. These data suggest that transcriptional activation by the Rtg1p-Rtg3p complex is largely the function of Rtg3p. Experiments are also presented suggesting that Rtg3p is limiting for gene expression in respiratory-competent (p°) cells.

Eukaryotic cells are able to monitor and respond to changes in mitochondrial function through changes in nuclear gene expression (1–5). We have termed this pathway retrograde regulation, and have suggested that it operates as a homeostatic mechanism for accommodating various cellular activities to alterations in mitochondrial function (6). One example of retrograde regulation in the yeast Saccharomyces cerevisiae is the elevated expression of the CIT2 gene in cells with dysfunctional mitochondria (7). CIT2 encodes a peroxisomal isoform of citrate synthase (8, 9), which functions in the glyoxylate cycle in one of two steps that utilize acetyl coenzyme A. The glyoxylate cycle enables cells to carry out a net conversion of acetate, available either as a sole carbon source or produced via β-oxidation, to carbohydrate by the shuttling of glyoxylate cycle intermediates (e.g. succinate) from the peroxisomes to mitochondria (10). Under conditions where the activity of the tricarboxylic acid (TCA) cycle is limiting, an increased activity of the glyoxylate cycle would allow for a more efficient use of two carbon compounds through anaerobic pathways. We have found that CIT2 transcription is activated as much as 30-fold in cells that have blocks in the TCA cycle or are respiratory-deficient, for example, because of a complete loss of mitochondrial DNA (p° petites) (7, 11, 12).

We have identified three genes, RTG1, RTG2, and RTG3, that are required both for basal and retrograde-regulated expression of CIT2 (11, 13). Surprisingly, these genes are also required for oleic acid-induced peroxisomal proliferation and the induction of enzymes of the β-oxidation pathway (12, 14), both of which occur when oleic acid is present in the growth medium (15–17). Thus, the RTG genes appear to play a pivotal role in controlling metabolic interactions between mitochondria and peroxisomes.

RTG2 encodes a cytoplasmic protein containing an hsp70 type of ATP binding domain (18). Experiments to be presented elsewhere show that Rtg2p has properties of a heat shock protein that may function as a molecular chaperone in the control of gene expression. 2 RTG1 and RTG3 encode basic helix-loop-helix leucine zipper (bHLH/Zip) transcription factors (11, 13). Rtg1p has some unusual features for a bHLH protein; in addition to an unusually long loop domain of some 39 amino acid residues, it has a very short basic DNA binding domain that lacks certain conserved amino acids found in the basic region of most other bHLH proteins. Those residues are known to make specific base contacts in the major groove of target site DNA (19–21). Rtg3p, on the other hand, has the features of a typical bHLH/Zip protein, including the conserved amino acids in the basic region of the bHLH motif. Recently, we showed that Rtg1p and Rtg3p bind, most likely as a heterodimer, to sequences in a 76-bp UAS element in the CIT2 promoter (13). The minimal DNA target site for binding of the Rtg1p-Rtg3p complex is not, however, an E box (22, 23), CANN1G, which is the core binding site for the great majority of bHLH transcription factors, but a novel sequence, GGTAC, which we have called an R box (19). The CIT2 UAS element contains two such

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1 The abbreviations used are: TCA, tricarboxylic acid; HLH, helix-loop-helix; bHLH/Zip, basic helix-loop-helix leucine zipper; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); UAS, upstream activation sequence; PAS, Per-Arnt-Sim.
2 L. Gopalakrishnan, unpublished results.
R boxes arranged as an inverted repeat separated by 28 bp of an AT-rich region. Both R box sites are required for full CIT2 expression and act synergistically in vivo (13).

In previous experiments, Rtg1p was tested for its ability to activate transcription by fusing to it the DNA binding domain of Gal4p (24). In wild-type cells, the Gal4-Gal4p chimera was able to activate transcription of an integrated LacZ reporter gene under the control of a GAL1 promoter element (UASG). Moreover, the activity showed a typical retrograde response, i.e., expression was greater in \( \rho^+ \) petites than in \( \rho^- \) cells. Transcriptional activation by Gal4-Gal4p-Rtg1p was absolutely dependent upon the presence of Rtg2p and at least one other protein, which we suggested was a bHLH protein that interacted with Rtg1p. That protein was subsequently identified as Rtg3p (13). To characterize further the role of Rtg1p and Rtg3p in the control of CIT2 expression, we have made a variety of Gal4p fusions to Rtg3p. Here we show that Rtg3p contains at least two transcriptional activation domains that can function independently of Rtg1p and Rtg2p. We have also identified an apparent inhibitory domain of Rtg3p transactivation rich in serine and threonine residues that could be potential phosphorylation sites of the protein. We suggest that Rtg3p may be limiting for CIT2 expression in respiratory-competent (\( \rho^+ \)) cells and that Rtg2p could function to recruit Rtg3p for interaction with Rtg1p.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—The *S. cerevisiae* strains used in this study are derivatives of either COP161 U7 (MATa ade1 lys1 ura3) (11) or SFY526 (MATa ade2 lys2 leu2 his3 trp1 gal4 gal80 Gal1-lacZ:Ura3). SFY526/lrtg1 was described previously (24). SFY526/lrtg2 and SFY526/lrtg3 are derivatives of SFY526, in which the reading frame encoding amino acids 23–573 of Rtg2p and the reading frame encoding amino acids 175–340 of Rtg3p were replaced, respectively, with the HIS3 gene. All strains exist both as a respiratory-competent \( \rho^+ \) cells and respiratory-incompetent \( \rho^- \) derivatives lacking mtDNA. The \( \rho^- \) strains were obtained by growth on rich dextrose medium containing 20 \( \mu \)g/ml ethidium bromide. Each \( \rho^- \) derivative was stained with 4',6-diamino-2-phenyldimethidole to ensure that no mitochondriam DNA was present. Cells were grown on rich yeast medium (1% yeast extract; 2% Bacto-peptone) containing 2% glucose (YPD); 2% raffinose (YPF); or 3% glycerol (YPG) at 30 °C. Plasmids were selected for by growth on minimal YNB medium (0.67% yeast nitrogen base without amino acids and 2% dextrose (YNBD), 2% raffinose (YNBR), or 3% glycerol (YNBG) supplemented with 1% casamino acids or individual amino acids as required.

Recombinant Plasmids—Standard molecular biology techniques were used for the construction, amplification, and manipulation of plasmids (25). The vector pGB9T (2\( \mu \), TRP3, GenBank accession no. U07646) was used to construct fusions to the Gal4p DNA binding domain. The vector pGAD424 (2\( \mu \), LEU2, GenBank accession no. U07647) was used to construct fusions to the Gal4p activation domain. The RTG1 fusion plasmid p2H26–177 was described previously (24). pGAD-RTG1 encodes the Gal4p activation domain fused to amino acids 26–177 of Rtg1p. PCR primers, containing a 5' EcoRI and 3' PstI site, were used to construct the series of GAL4–RTG3 fusion plasmids displayed in Fig. 1 and pGAD-RTG3 (encoding the full-length Rtg3p fused to the Gal4p activation domain). pGBT-RTG2 encodes the Gal4p DNA binding domain fused to full-length Rtg2p. The sequence of all PCR generated constructs was verified using the Sequenase kit (U. S. Biochemical Corp.).

A 1.5-kb Kpn1-BamHI genomic RTG1 fragment was cloned into pRS416 (CEN, URA3) and the multicopy vector yaplac195 (2\( \mu \), URA3) to give pRTG1–416 and p195-RTG1. A 3.1-kb genomic Xba1-BamHI RTG3 fragment was cloned into pRS416, yaplac195, and yaplac181 (2\( \mu \), LEU2) to give pRTG3–416, p195-RTG3, and p181-RTG3.

Yeast Transformation, Cell Extracts, and \( \beta \)-Galactosidase Assays—Yeast were transformed using lithium acetate and heat shock (26). Transformants carrying the desired plasmid or plasmid pairs were selected on YNB supplemented with the necessary amino acids. Liquid precultures were inoculated with a pool of 15–20 independent transformants and grown in selective YNBp (\( \rho^- \)) or YNBp (\( \rho^+ \)). Selective YNBR cultures were inoculated at a low density (0.01 \( A_{600} \)), grown at 30 °C and harvested at mid-log phase (0.9 \( A_{600} \)). Cell extracts and assays were carried out as described by Rose and Botstein (27). For each plasmid-strain combination, these extracts were assayed in triplicate.

Northern Blot Analysis—COP161 U7 \( \rho^+ \) and \( \rho^- \) strains were transformed with an empty control plasmid, or plasmid carrying RTG1 or RTG3 (either 2\( \mu \) or CEN). Selective YNBp (\( \rho^- \)) or YNBRp (\( \rho^+ \)) precultures were used to inoculate selective YNBR cultures at a low density (0.01 \( A_{600} \)). Cells were harvested at mid-log phase and total RNA was isolated (28). RNA was fractionated on an agarose gel and probed for actin and CIT2 mRNA abundance as described previously (7). Signals were quantified using a Molecular Dynamics PhosphorImager.

RESULTS

Transactivation by Gal4-Gal4p Is Dependent on Rtg2p and Rtg3p—In previous experiments, we showed that a chimeric protein consisting of the DNA binding domain of Gal4p fused to Rtg1p was able to activate expression of an integrated LacZ reporter gene under the control of a Gal4p-responsive UASG element (24). However, those studies revealed that Rtg1p by itself could not transactivate, but appeared to require, in addition to Rtg2p, one or more proteins for transcriptional activity. That conclusion was strengthened by the identification of a new bHLH/Lip protein encoded by the RTG3 gene, which is also essential for CIT2 expression, and the finding that Rtg3p binds with Rtg1p at two sites in the CIT2 UASG (13). To determine whether Rtg3p is also required for transactivation mediated by the Gal4-Rtg1p chimeric protein, where Rtg1p is directed to the UASG element in the reporter gene solely by the Gal4p DNA binding domain, SFY526/lrtg3 cells were transformed with p2H26–177 encoding the Gal4p DNA binding domain fused directly to the HLH domain of Rtg1p (Fig. 1A). The \( \beta \)-galactosidase activity of this transformant was then compared with p2H26–177 transformants of wild-type SFY526 cells and its \( \rho^+ \) and \( \rho^- \) derivatives.

Fig. 2 shows that there is a retrograde response in the transactivation by p2H26–17 in SFY526 transformants, i.e. activity was greater in respiratory-deficient \( \rho^- \) petites than in \( \rho^+ \) cells of that strain. In addition, activity both in \( \rho^+ \) and \( \rho^- \) cells was comparably greater in SFY256/lrtg1 cells, presumably because endogenous Rtg1p was not present to compete for limited interacting proteins required for transactivation of the Gal4-Rtg1p fusion protein. In contrast to these results, no transcriptional activation was observed in either SFY256/lrtg2 or SFY256/lrtg3 transformants. Altogether, regulation of expression of the UASG/LacZ reporter gene by Gal4-Rtg1p resembles transcriptional control of the CIT2 gene, whereby expression is elevated in \( \rho^- \) cells, and is dependent on RTG1 and RTG3 in both \( \rho^- \) and \( \rho^+ \) cells (11, 13). These results are consistent with the notion that transactivation occurs via an Rtg1p-Rtg3p heterodimeric complex, and that Rtg3p contains one or more transactivation domains.

Although Rtg2p is required for Gal4-Rtg1p-mediated transactivation of the UASG/LacZ reporter gene, it is unlikely to be involved directly in transcriptional activation. First, we have fused full-length Rtg2p to the Gal4p DNA binding domain and found that this construct (pGBT-RTG2) by itself did not transactivate UASG/LacZ reporter gene expression in wild-type cells (Table I, part A). Second, in a standard two-hybrid experiment testing protein-protein interactions (29), when pGBT-RTG2, encodes the Gal4p DNA binding domain fused to Rtg2p, was co-transformed with either pGAD-RTG1 or pGAD-RTG3 encoding, respectively, Rtg1p or Rtg3p fused to the Gal4p activation domain, no increase in reporter gene expression was detected. This result indicates that there is no direct interaction between Rtg2p and either Rtg1p or Rtg3p.

Gal4-Rtg3p Can Transactivate UASG/LacZ Reporter Gene Independent of Rtg1p or Rtg2p—The results of the previous experiments suggest that, for transactivation of the UASG/LacZ reporter gene, either there is a necessary interaction
between Rtg1p and Rtg3p, or Rtg3p by itself is sufficient for transcriptional activation. To distinguish between these possibilities, we constructed a series of chimeric proteins consisting of the Gal4p DNA binding domain fused to various domains of Rtg3p, including the full-length protein (Fig. 1B). These constructs were then tested for their ability to transactivate expression of the UAS G-LacZ reporter gene in wild-type and mutant SFY526 cells. Fig. 3 shows that the full-length Gal4-Rtg3p chimera, p3GB1–486 (Fig. 1B), could transactivate in wild-type cells. Unlike Gal4-Rtg1p, however, transactivation did not show a typical retrograde response of higher activity in \( r^\circ \) cells. Moreover, Gal4-Rtg3p could transactivate in all three \( \Delta rtg \) mutant backgrounds as well as, or better than, in wild-type cells. Except for expression in \( \Delta rtg1 \) cells, activity was lower in the \( r^\circ \) petite than in \( r^1 \) cells. These results are in sharp contrast to Gal4-Rtg1p transactivation, which requires both Rtg2p and Rtg3p and which consistently shows a greater activity in \( r^\circ \) cells. From these experiments, we conclude that Rtg3p itself contains one or more domains responsible for transcriptional activation, but these are not by themselves responsive to retrograde control.

Rtg3p Contains Independent Amino- and Carboxyl-terminal Activation Domains—To define the activation domain(s) of Rtg3p, we constructed a series of Gal4-Rtg3p fusions linking different portions of the protein to the DNA binding domain of Gal4p. Cultures were inoculated with a pool of 15–20 transformants and grown to mid-log phase on selective YNBR medium. \( \beta \)-Galactosidase assays were done in triplicate as described by Rothermel et al. (24), and the activities are expressed as nanomoles/min/mg of protein. Between Rtg1p and Rtg3p, or Rtg3p by itself is sufficient for transcriptional activation. To distinguish between these possibilities, we constructed a series of chimeric proteins consisting of the Gal4p DNA binding domain fused to various domains of Rtg3p, including the full-length protein (Fig. 1B). These constructs were then tested for their ability to transactivate expression of the UAS G-LacZ reporter gene in wild-type and mutant SFY526 cells. Fig. 3 shows that the full-length Gal4-Rtg3p chimera, p3GB1–486 (Fig. 1B), could transactivate in wild-type cells. Unlike Gal4-Rtg1p, however, transactivation did not show a typical retrograde response of higher activity in \( r^\circ \) cells. Moreover, Gal4-Rtg3p could transactivate in all three \( \Delta rtg \) mutant backgrounds as well as, or better than, in wild-type cells. Except for expression in \( \Delta rtg1 \) cells, activity was lower in the \( r^\circ \) petite than in \( r^1 \) cells. These results are in sharp contrast to Gal4-Rtg1p transactivation, which requires both Rtg2p and Rtg3p and which consistently shows a greater activity in \( r^\circ \) cells. From these experiments, we conclude that Rtg3p itself contains one or more domains responsible for transcriptional activation, but these are not by themselves responsive to retrograde control.

**Rtg3p Contains Independent Amino- and Carboxyl-terminal Activation Domains**—To define the activation domain(s) of Rtg3p, we constructed a series of Gal4-Rtg3p fusions linking different portions of the protein to the DNA binding domain of Gal4p (Fig. 1B). These constructs were then tested for activity in \( r^\circ \) and \( r^\circ \) derivatives of strain SFY526. The results of these experiments (Fig. 4) show, first, that there are at least two independent activation domains in Rtg3p, a strong COOH-terminal domain (amino acids 375–486) and a weaker NH2-terminal domain (amino acids 1–147 of the Gal4p DNA binding domain).
terminal domain (amino acids 1–175). Second, neither of these domains contain the HLH/Zip protein dimerization motif, indicating that transactivation does not require an HLH/Zip-mediated protein-protein interaction. Although there was somewhat greater activity in construct pGB300–486 containing the HLH/Zip domain, these results, nevertheless, contrast sharply with those obtained with the Gal4-Rtg1p fusions, where the HLH/Zip domain was found to be essential for activity in UASG-LacZ reporter gene expression (24).

A third significant outcome of these experiments is that the region of Rtg3p from amino acids 176 to 282 was not only inactive in supporting reporter gene expression but also appeared to be inhibitory to the activity of the NH2- and COOH-terminal activation domains. For example, construct p3GB1–282 was less active than construct p3GB1–175, and construct p3GB176–286 was considerably less active than p3GB300–486 or p3GB375–486 (Figs. 1B and 4). It is noteworthy that the inactive, and apparently inhibitory, domain of Rtg3p from amino acids 176–282 is very rich in serine and threonine residues, and thus may be potentially important as phosphorylation sites in the protein. Finally, as is the case with the full-length fusion construct, all of the active, truncated Gal4-Rtg3p fusions were also active in Δrtg1, Δrtg2, and Δrtg3 cells (data not shown). From these data, we conclude that Rtg3p contains NH2- and COOH-terminal transactivation domains, which, when directed to the Gal4p DNA binding site, do not require the bHLH/Zip dimerization domain or the products of the two other known Rtg genes for activity.

Rtg1p and Rtg3p Can Interact in the Absence of Rtg2p—The findings presented thus far suggest that Rtg3p is the functional transactivator of the Rtg1p-Rtg3p complex. Hence, reporter gene expression mediated by Gal4-Rtg1p would require recruitment of Rtg3p and interaction of these proteins via their bHLH/Zip motifs. Gal4-Rtg3p, however, which contains independent transactivation domains, would not require Rtg1p for transactivation of reporter gene expression from a UASG element. What then is the function of Rtg2p in transactivation? Rtg2p has no known DNA binding motifs, but it does contain an hsp70-like ATP binding domain (18) and has some properties of a heat shock protein and molecular chaperone,2 such as a peptide-stimulated ATPase activity (30). Thus, Rtg2p might function to promote a direct interaction between Rtg1p and Rtg3p. To examine whether Rtg1p and Rtg3p can interact in the absence of Rtg2p when both proteins are targeted to the nucleus via plasmid-encoded heterologous nuclear localization signals, we used the construct, p2H26–177, which fuses the Gal4p DNA binding domain to Rtg1p, in a two-hybrid assay together with a second plasmid, pGAD-RTG3, encoding full-length Rtg3p linked to the Gal4p activation domain. The two constructs, p2H26–177 and pGAD-RTG3, were examined singly (along with the control plasmids) and in combination for their ability to transactivate the UASG-LacZ reporter gene in p+ and p derivatives of wild-type, Δrtg3, and Δrtg2 SFY526 cells.

Table I, part B, shows that, as observed previously (24), the construct encoding the Gal4p DNA binding domain fused to Rtg1p (p2H26–177) was able to transactivate the reporter gene in wild-type SFY526 cells in a retrograde-responsive manner. By contrast, no transactivation was observed in cells transformed with pGAD-RTG3, which encodes the Gal4p activation domain fused to Rtg3p. However, in wild-type cells transformed with both p2H26–177 and pGAD-RTG3, a high level of transactivation was observed that was 2–3.6-fold greater than with p2H26–177 alone. In Δrtg3 cells, neither p2H26–177 or pGAD-RTG3 alone showed any activity, but activity was observed in cells transformed with both plasmids, indicating an interaction between Rtg1p and Rtg3p. In Δrtg2 cells, some transactivation by p2H177 was observed but the activity was only a fraction (10% or less) of that seen in wild-type cells. Importantly, strong transactivation was observed in Δrtg2 cells containing both p2H26–177 and pGAD-RTG3, indicating that Rtg2p is not required for the direct interaction between Rtg1p and Rtg3p.

These findings are consistent with previous observations that an Rtg1p-Rtg3p dependent complex is formed in electrophoretic mobility shift assays of the 76-bp CIT2 UAS, using whole cell extracts from a Δrtg2 strain (11) and that recombinant Rtg1p and Rtg3p can interact and bind to their target DNA sites in vitro (13). Finally, it is interesting to note that the response of the p2H26–177/pGAD-RTG3 pair in all cases showed an elevated expression in p+ cells, suggesting that some component of the retrograde response is post-transcriptional, since both proteins were over expressed from constitutive promoters.

Overexpression of RTG3—Previous experiments showed that the level of Rtg1p detected by Western blot analysis does not differ significantly between p+ and p cells (24), suggesting that the CIT2 retrograde response is unlikely to be controlled by a change in the level of Rtg1p. We have not been able to determine whether the CIT2 retrograde response might be con-
TABLE I

Two-hybrid analysis of interactions among Rtg1p, Rtg2p, and Rtg3p

| Gal4p domain | DNA binding | activation | strain | ρ⁺ | ρ⁻ |
|--------------|-------------|------------|--------|---|---|
| A.           |             |            |        |   |   |
| pGBT-RTG2    | pGAD412     | WT         | 3.5 ± 1.6 | 2.0 ± 1 |
| pGBT-RTG2    | pGAD-RTG1   | WT         | 3.7 ± 0.6 | 4.2 ± 0.4 |
| pGBT-RTG2    | pGAD-RTG3   | WT         | 2.3 ± 0.5 | 1.5 ± 0.8 |
| B.           |             |            |        |   |   |
| p2H26–177    | pGAD412     | WT         | 2.45 ± 0.5 | 1357 ± 4 |
| pGDT9        | pGAD-RTG3   | WT         | 3.4     | 2.8 |
| p2H26–177    | pGAD-RTG3   | WT         | 577 ± 3 | 4873 ± 37 |
| p2H26–177    | pGAD412     | Δrtg3      | 4.2 ± 1.3 | 3.2 ± 1.6 |
| pGDT9        | pGAD-RTG3   | Δrtg3      | 2.5 ± 2.5 | 2.2 ± 1.9 |
| p2H26–177    | pGAD-RTG3   | Δrtg3      | 181 ± 3 | 447 ± 8 |
| p2H26–177    | pGAD412     | Δrtg2      | 22 ± 0.8 | 47 ± 1.2 |
| pGDT9        | pGAD-RTG3   | Δrtg2      | 2.1 ± 1 | 3.4 ± 2.4 |
| p2H26–177    | pGAD-RTG3   | Δrtg2      | 402 ± 14 | 766 ± 13 |

ACTIVITY is measured in nanomoles/min/mg protein. pGBT9-RTG2 encodes the Gal4p DNA binding domain fused to amino acid full-length Rtg2p. p2H26–177 encodes the Gal4p DNA binding domain fused to full-length Rtg1p. p2H26–177 and p2H26–177Δrtg2 multisite plasmids. In all cases, expression of RTG1 and RTG3 was under control of their own promoters. Total cellular RNA was isolated from log-phase cultures grown on YNBR + casamino acids medium and analyzed by Northern blotting to determine CIT2 expression relative to an internal control for actin mRNA (Fig. 5). The data were quantified by PhosphorImager analysis, and the relative amount of CIT2 mRNA, normalized to the level of actin mRNA, is present below each lane.

In this series of experiments, the retrograde response for CIT2 expression (p⁺/p⁻) was 5.6 (Fig. 5, lanes 1 and 2). In p⁺ cells, overexpression of Rtg1p from either the centromere or 2μ-based plasmid resulted in a less than 3-fold increase in CIT2 mRNA abundance and little or no increase in p⁻ cells (lanes 3–6). There was a somewhat larger increase in CIT2 mRNA when RTG3 was overexpressed in p⁺ cells from the centromere plasmid (lane 7), and nearly a 10-fold increase in when RTG3 was overexpressed from the 2μ-based plasmid (lane 9). In p⁻ cells, overexpressing RTG1 or RTG3 from either vector led only to a relatively small increase in CIT2 expression above the control cells (compare lane 2 with lanes 4, 6, 8, and 10). These data suggest, first, that the retrograde response is effectively limited by Rtg3p and less so by Rtg1p, and second, that CIT2 transcription in p⁻ cells is close to the limit of expression that is regulated by the Rtg1p-Rtg3p heterodimeric complex.

DISCUSSION

Components of the CIT2 Transcription Apparatus—Transcription of the CIT2 gene requires the bHLH/Zip proteins, Rtg1p and Rtg3p, which bind as heterodimers to two identical, non-E box target sites (R boxes, GGTCAC) in the UASr element of the CIT2 promoter (13); neither protein alone is able to bind to the R box in vitro or to activate CIT2 transcription in vivo. CIT2 transcription also requires Rtg2p, a protein with no obvious DNA binding motifs, that is related to the hsp70 family of heat shock proteins (18). Based on two-hybrid analysis carried out in the current study, we conclude that Rtg2p neither binds to Rtg1p or Rtg3p nor is required for the direct interaction of these transcription factors with each other.

Transcription by Rtg3p—To understand better how the Rtg proteins function both in basal and retrograde regulated expression of the CIT2 gene, we have extended our previous analysis of the transcriptional activity of fusion proteins between the Gal4p DNA binding domain and Rtg1p (24) to include a similar analysis of Gal4-Rtg3p chimeras; these were analyzed in wild-type p⁺ and p⁻ cells and in different rtg mutant backgrounds. Such fusions allow an assessment of the transcriptional activity of Rtg3p and its various domains without requiring DNA binding to endogenous target sites. Our experiments show that Gal4-Rtg1p fusion proteins require Rtg3p and Rtg2p to activate transcription of a lacZ reporter gene. By contrast, Gal4-Rtg3p fusion proteins can transactivate reporter gene expression in the absence of either Rtg1p or

centromere-based (pRS426) or 2μ-based multicopy (yplac195) plasmids. In all cases, expression of RTG1 and RTG3 was under control of their own promoters. Total cellular RNA was isolated from log-phase cultures grown on YNBR + casamino acids medium and analyzed by Northern blotting to determine CIT2 expression relative to an internal control for actin mRNA (Fig. 5). The data were quantified by PhosphorImager analysis, and the relative amount of CIT2 mRNA, normalized to the level of actin mRNA, is present below each lane.

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DISCUSSION

Components of the CIT2 Transcription Apparatus—Transcription of the CIT2 gene requires the bHLH/Zip proteins, Rtg1p and Rtg3p, which bind as heterodimers to two identical, non-E box target sites (R boxes, GGTAC) in the UASr element of the CIT2 promoter (13); neither protein alone is able to bind to the R box in vitro or to activate CIT2 transcription in vivo. CIT2 transcription also requires Rtg2p, a protein with no obvious DNA binding motifs, that is related to the hsp70 family of heat shock proteins (18). Based on two-hybrid analysis carried out in the current study, we conclude that Rtg2p neither binds to Rtg1p or Rtg3p nor is required for the direct interaction of these transcription factors with each other.

Transcription by Rtg3p—To understand better how the Rtg proteins function both in basal and retrograde regulated expression of the CIT2 gene, we have extended our previous analysis of the transcriptional activity of fusion proteins between the Gal4p DNA binding domain and Rtg1p (24) to include a similar analysis of Gal4-Rtg3p chimeras; these were analyzed in wild-type p⁺ and p⁻ cells and in different rtg mutant backgrounds. Such fusions allow an assessment of the transcriptional activity of Rtg3p and its various domains without requiring DNA binding to endogenous target sites. Our experiments show that Gal4-Rtg1p fusion proteins require Rtg3p and Rtg2p to activate transcription of a lacZ reporter gene. By contrast, Gal4-Rtg3p fusion proteins can transactivate reporter gene expression in the absence of either Rtg1p or
Transactivation by Rtg3p

**Fig. 5.** The influence of increased copy number of RTG1 and RTG3 on CIT2 mRNA levels in ρ⁺ and ρ⁻ cells. COP611U7 ρ⁺ and ρ⁻ cells were transformed with a low copy centromere-based vector (pRS416) or a high copy 2μ-based vector (pplac195), both with a URA3 marker, and an insert containing either the RTG1 or RTG3 gene. The ρ⁺ and ρ⁻ control cells were transformed with the empty pRS416 plasmid. Total RNA was isolated from mid-log phase cultures grown on selective YNB + casamino acids as described under “Materials and Methods.” The blots were hybridized with probes specific for CIT2 and ACT1 as described previously (11). The hybridization signals were quantified using a Molecular Dynamics PhosphorImager and the ratio of the relative signals for CIT2 and ACT1 are indicated below the figure.

**A Potential Regulatory Domain of Rtg3p**—A central domain of Rtg3p from amino acids 176 to 282 that was tested for transcriptional activity was not only inactive but also inhibitory to activation by other domains; constructs lacking that central domain had significantly more activity than the constructs containing it (for example, p3GB1–282 versus p3GB1–175 shown in Fig. 4). This apparently inhibitory domain of Rtg3p is very rich in serine and threonine residues and thus could contain potential phosphorylation sites that might play a role in regulating the activity of the Rtg1p-Rtg3p complex. That phosphorylation can play a role in regulating the activity of bHLH proteins is suggested from studies showing that there is a dimerization-dependent phosphorylation of the muscle-specific bHLH transcription factors myogenin (35) and MyoD (36). The potential for a direct regulatory role of this phosphorylation is suggested by the finding that mutations of the dimerization-dependent phosphorylation sites enhance the transcriptional activity of myogenin heterodimeric complexes (35). It will be of interest to learn whether the serine/threonine-rich region of Rtg3p is a target for phosphorylation of the protein that might affect the transcriptional activity of the Rtg1p-Rtg3p complex.

The dioxin receptor, also known as the aryl hydrocarbon receptor, is another example of a bHLH protein with a regulatory domain that may bear on the activity of the Rtg1p-Rtg3p complex. The dioxin receptor is a ligand-responsive member of the bHLH/PAS family, which forms a heterodimer with the bHLH/PAS protein, Arnt, and binds to specific xenobiotic-responsive elements regulating transcription of genes involved in drug metabolism (37–40). The dioxin receptor has been shown to have a strong COOH-terminal transactivation domain, which is attenuated by a central 82-amino acid ligand binding domain of the protein (41). It is interesting that this inhibitory domain of the dioxin receptor is a site where a regulator molecule, an hsp90, interacts to prevent dimerization with Arnt (42, 43).

We suggest that Rtg3p, which is a low abundance protein both in ρ⁺ and ρ⁻ cells, is recruited to the R box through its interaction with the more abundant and constitutively expressed Rtg1p. Although the details of how the complex contacts the R box sites remain to be established, it is likely that Rtg3p interacts with the CA dinucleotide of the R box because the basic region of the Rtg3p bHLH/Zip motif contains the critical conserved amino acid residues, such as an Arg, His, and Glu, with characteristic spacing that have been shown for other bHLH proteins to make specific contacts with the CA dinucleotide of the CANNTG E box half-site in the major groove of DNA (19, 21, 44). These key amino acid residues are not present as such in the truncated basic domain of Rtg1p. It is also significant that the Rtg1p-Rtg3p complex does not bind in vitro to common E box sites that were tested under conditions where robust binding was observed to the R box (13). Thus, the unique structural characteristics of Rtg1p may be responsible for determining the unusual DNA binding specificity of this bHLH/Zip complex.

What is the role of Rtg2p in the transcriptional process mediated by Rtg1p-Rtg3p? Since overexpression of Rtg3p can elevate target gene expression in ρ⁻ cells, it is conceivable that Rtg2p could function to modulate the expression of RTG3. This is unlikely, however, for a number of reasons. First, we have not detected any effect of an rtg2 null allele on the level of expression of RTG3 mRNA (data not shown). Second, the abundance of RTG3 mRNA does not vary by more than 2-fold between ρ⁻ and ρ⁺ cells (13). And finally, electrophoretic mobility shift assays using whole cell extracts from a Δrtg2 mutant strain, in which there is no CIT2 expression, yield the same Rtg1p-Rtg3p-dependent complex with a CIT2 UAS, probe, as do extracts from wild-type cells (11, 13). An alternative possibility suggested by our data is that in ρ⁻ cells, Rtg3p is sequestered in an inactive form by one or more proteins, and that Rtg2p functions to regulate the availability of Rtg3p for transcriptional activation. This would be analogous to the sequestration of transcription factors such as NF-kB by I-kB (reviewed by Verma et al. (45)), the E protein family of bHLH proteins by Id (46) and the bHLH/PAS dioxin-responsive and related Sim family of transcription factors by hsp90 (43, 47, 48).
Overexpressing Rtg3p in ρ− cells, in which the majority of the endogenous protein might be sequestered in an inactive form, could result in an increase in gene expression by effectively titrating out the protein(s) responsible for the sequestration. The finding that overexpression of Rtg1p also resulted in some increase in CIT2 expression in ρ− cells, but to a lesser extent than was observed with Rtg3p, could be accounted for by a competition for the free and sequestered forms of Rtg3p. The presence of an hsp70-like ATP binding domain in Rtg2p (18) and observations that Rtg2p has the properties of a heat shock protein raise the possibility that Rtg2p could function as a molecular chaperone to promote the formation of the Rtg1p-Rtg3p heterodimer for transcriptional activation.

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