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A Basic Helix-Loop-Helix–Leucine Zipper Transcription Complex in Yeast Functions in a Signaling Pathway from Mitochondria to the Nucleus

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The expression of some nuclear genes in *Saccharomyces cerevisiae*, such as the *CIT2* gene, which encodes a glyoxylate cycle isoform of citrate synthase, is responsive to the functional state of mitochondria. Previous studies identified a basic helix-loop-helix–leucine zipper (bHLH/Zip) transcription factor encoded by the *RTG1* gene that is required for both basal expression of the *CIT2* gene and its increased expression in respiratory-deficient cells. Here, we describe the cloning and characterization of *RTG3*, a gene encoding a 54-kDa bHLH/Zip protein that is also required for *CIT2* expression. Rtg3p binds together with Rtg1p to two identical sites oriented as inverted repeats 28 bp apart in a regulatory upstream activation sequence element (UAS_r) in the *CIT2* promoter. The core binding site for the Rtg1p-Rtg3p heterodimer is 5′-GGTCAC-3′, which differs from the canonical E-box site, CANNTG, to which most other bHLH proteins bind. We demonstrate that both of the Rtg1p-Rtg3p binding sites in the UAS_r element are required in vivo and act synergistically for *CIT2* expression. The basic region of Rtg3p conforms well to the basic region of most bHLH proteins, whereas the basic region of Rtg1p does not. These findings suggest that the Rtg1p-Rtg3p complex interacts in a novel way with its DNA target sites.

Cells of the yeast *Saccharomyces cerevisiae* are able to monitor and respond to changes in mitochondrial function through accommodating changes in nuclear gene expression (26). We have referred to this process as retrograde regulation, which can be thought of as a stress response to mitochondrial dysfunctions (reviewed by Shyjan and Butow [36]). One example of retrograde regulation is the elevated expression of the *CIT2* gene in response to various mitochondrial lesions (17). *CIT2* encodes a peroxisomal isoform of citrate synthase (CS2) (15, 21) that functions in the glyoxylate cycle. CS2 has 83% sequence similarity with the tricarboxylic acid (TCA) cycle isoform of citrate synthase, CS1, encoded by the *CIT1* gene. Various mitochondrial lesions, such as a block in the TCA cycle or the loss of mitochondrial DNA, result in a transcriptional activation of *CIT2* anywhere from 2- to 30-fold. This activation is dependent, in part, on the kind mitochondrial defect (4, 17). We have suggested that the corresponding increases in CS2 activity could compensate for decreases in TCA cycle activity (17) through the known metabolic interactions between the glyoxylate and TCA cycles (38).

We have identified two genes, *RTG1* and *RTG2*, that are central to this novel signaling pathway (16). Each is required for basal as well as retrograde-regulated *CIT2* expression. Surprisingly, both *RTG1* and *RTG2* are also essential for peroxisome proliferation (4, 13), which can be induced in yeast cells by the presence of oleic acid in the growth medium (14, 37, 40). *RTG1* and *RTG2* are also required for the oleic acid-dependent increase in expression of *POX* genes (encoding enzymes of the β-oxidation pathway) and *PMP27*, which encodes a protein associated with peroxisomal membranes (20). However, unlike the case for the *CIT2* gene, there is no indication that the promoter activities of these other genes encoding peroxisomal proteins are directly influenced by the *RTG* genes. We have suggested that the *RTG* genes may act early in the pathway of oleic acid-induced biogenesis of peroxisomes (4). This provocative link between mitochondria, peroxisomes, and the nucleus suggests that retrograde regulation is a fundamental process of organelle communication that functions to coordinate metabolic activities in response to changes in the functional state of mitochondria.

RTG1 encodes a 19-kDa protein that is a member of the basic helix-loop-helix–leucine zipper (bHLH/Zip) family of transcription factors (7, 23–25). Proteins of this class contain (i) a basic DNA binding domain followed by two helix regions separated by a loop of variable length and (ii) a leucine zipper domain that contributes an interface for dimerization. Rtg1p is unusual for a number of reasons: it has a truncated basic domain compared with most other members of the bHLH/Zip family of proteins; and its basic domain also lacks a number of conserved amino acid residues that, from crystal structure analysis of several bHLH/Zip proteins, function in contacting the canonical E-box DNA target site for bHLH proteins, CANNTG (6, 7, 19). Rtg1p also has an unusually long loop domain (some 39 amino acids) separating the two helical regions. Finally, Rtg1p binds to DNA sequences contained within a 76-bp regulatory upstream activation sequence (UAS_r) element in the 5′ flanking region of *CIT2* that are necessary and sufficient for basal and retrograde-regulated expression of *CIT2* but which lack an E box. Thus, Rtg1p is likely to interact in a novel way with its *CIT2* UAS_r target site.

bHLH and bHLH/Zip transcription factors activate gene expression as either homo- or heterodimers through oligomerization interfaces contained in the HLH and Zip domains (reviewed in reference 5). We have recently shown that a chimeric protein, in which the DNA binding domain of the transcriptional regulator protein GAL4 was fused to Rtg1p,

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can transactivate expression of a reporter gene construct containing the GAL4 target site (UAS_G) fused to the *Escherichia coli lacZ* gene (32). Moreover, the GAL4-Rtg1p fusion transactivated in a retrograde-responsive manner; i.e., activity was greater in respiratory-incompetent [*rho*^{0}] cells lacking mitochondrial DNA than in wild-type [*rho*$^+$] cells. An important conclusion from those studies was that Rtg1p does not itself contain an activation domain but must interact with an unidentified protein, most likely another bHLH protein, which together are required for [*rho*$^+$]-[*rho*^{0}] retrograde-responsive *CIT2* expression. Analysis of the *RTG2*-encoded protein (Rtg2p) and DNA binding studies with extracts from cells lacking Rtg2p (16) suggest that Rtg2p, whose function is unknown, is not likely to be a transcription factor and thus not the Rtg1p activation partner.

Here we describe the cloning and characterization of a third gene, *RTG3*, required for *CIT2* expression. *RTG3* encodes a 54-kDa bHLH/Zip protein. Unlike Rtg1p, Rtg3p contains those amino acid residues in its basic region that are conserved among most other bHLH proteins and which are known to contact specific bases in an E-box DNA target site. Nevertheless, we show that Rtg1p and Rtg3p are required together to bind to each of two identical non-E-box core sequences, 5′-GGTCAC-3′, in the 76-bp *CIT2* UAS_r. These sites, which are duplicated as an inverted repeat separated by 28 bp of an AT-rich sequence, act synergistically to effect *CIT2* expression in vivo.

MATERIALS AND METHODS

Yeast strains and growth conditions. The *S. cerevisiae* strains used in this study are derivatives of strain COP161 U7 (*MAT*α *ade1 lys1 ura3*). The Δ*rtg1* and Δ*rtg2* gene replacement strains were described previously (16). Ethyl methanesulfonate mutagenesis was carried out as previously described (16) to generate the mutant *rtg3-1*. A *lacZ* reporter gene, in which 607 bp of the 5′ flanking region of *CIT2* fused to the *E. coli lacZ* gene, was integrated into the *URA3* locus as described by Liao and Butow (16). A Δ*rtg3* strain was made by replacing codons 175 to 340 with the *URA3* gene by standard transformation procedures (31) and confirmed by Southern blot analysis. All strains exist as [*rho*$^+$] and [*rho*^{0}] derivatives. The [*rho*^{0}] derivatives were obtained by several passages of [*rho*$^+$] cells through YPD medium containing 20 μg of ethidium bromide per ml. Cells were grown at 30°C on YP medium (1% yeast extract, 2% Bacto Peptone) and either 2% glucose (YPD), 2% raffinose (YPR), or 2% glycerol (YPG) as a carbon source. Selective YNB medium contained 0.67% yeast nitrogen base without amino acids, 2% dextrose (YNBD), 2% raffinose (YNBR), or 2% potassium acetate (YNBAc) as a carbon source, supplemented with Casamino Acids or with individual amino acids as required.

Cloning and sequencing of *RTG3* and *rtg3-1*. Two plasmids encoding wild-type *RTG3* were identified from a genomic library, 8979-3A (30) as described in Results. *RTG3* was initially subcloned as a 4.2-kb fragment by phenotypic complementation. Two *Hin*dIII fragments of 1.4 and 2.8 kb were then subcloned into pUC19. A set of nested exonuclease III deletions was generated from each of the fragments. Both strands of each fragment were sequenced by using a Sequenase kit (U.S. Biochemical). The junction of the two fragments and the gaps among the deletions were sequenced by using oligonucleotide primers. To sequence the *rtg3-1* mutant allele, we cloned a 1.8-kb PCR fragment amplified from *rtg3-1* genomic DNA in pGem-3Zf(+) (Promega). Three independent clones were sequenced.

Northern blot and RNase protection analysis. Strains were precultured in selective medium to maintain either a control plasmid, YCp50, or YCp50 containing an insert of a genomic DNA fragment encoding *RTG3*. Precultures were used to inoculate YPR cultures at an optical density at 600 nm of ~0.02 and grown to mid-logarithmic phase. Total cellular RNA was isolated by the procedure of Schmitt et al. (34), fractionated on 1.2% agarose gels, and transferred to Nytran Plus membranes essentially as described in reference (33). *CIT2* and actin probes were labeled with [α-^{32}P]ATP by using a Random Primed DNA Labeling kit (Boehringer Mannheim). Blots were hybridized in 0.5 M sodium phosphate (pH 7.4)–7% sodium dodecyl sulfate–1% bovine serum albumin–1 mM EDTA at 65°C. Hybridization signals were quantified with a Molecular Dynamics PhosphorImager.

RNase protection analysis of *CIT2* and actin mRNA levels was carried out on total RNA preparations essentially as described by Liao and Butow (16). For *RTG3* mRNA analysis, a fragment encoding amino acids 375 through 486 of Rtg3p was cloned into the *Eco*RI and *Pst*I sites of pGEM-3Zf(+), using PCR primers to generate the required restriction sites. This plasmid was linearized with *Bst*EII and SP6 RNA polymerase was used to generate a 196-bp riboprobe. Total cellular RNA was isolated from log-phase cultures of COP161 U7 [*rho*$^+$] and [*rho*^{0}] cells grown on YPR.

Preparation of recombinant Rtg1p and Rtg3p. The open reading frames of *RTG1* and *RTG3* were amplified by PCR and cloned in frame into the pMAL-cII vector (New England Biolabs). The recombinant plasmids were expressed in *E. coli*, and the proteins were purified on a maltose affinity column as instructed by the manufacturer. The fusion proteins were cleaved with factor Xa. A sample of the products was fractionated on a sodium dodecyl sulfate-polyacrylamide gel and then stained with Coomassie blue to verify the cleavage products.

EMSA. Whole-cell extracts were prepared as described previously (16). Electrophoretic mobility shift assays (EMSAs) were carried out as described by Rothermel et al. (32). These assays are modified from those originally reported by Liao and Butow (16) so that two rather that three gel-shifted bands are observed when the 76-bp *CIT2* UAS_r probe is incubated with extracts from [*rho*$^+$] or from [*rho*^{0}] cells. The 76-bp *CIT2* UAS_r was amplified by PCR. Complementary oligonucleotides spanning the A and A′ sites (see Fig. 5A) were annealed to form the 21- and 23-bp double-stranded DNA fragments by standard procedures. Fragments were end labeled with ^{32}P, using T4 polynucleotide kinase (Boehringer Mannheim). DNA fragments were incubated with recombinant proteins or cell extracts in 10 mM Tris-HCl (pH 8.0)–100 mM KCl–4 μg of denatured single-stranded salmon sperm DNA–2 mM $MgCl_2$–5% glycerol at 25°C for 20 min. The reaction mixture was fractionated on a 5% polyacrylamide gel at 4°C for 2 h. The gel was dried and exposed to X-ray film. Oligonucleotides used for PCR and EMSA templates are listed in Table 1.

UAS_r-*lacZ* constructs and β-galactosidase assays. Plasmid p76AA′ contains the 76-bp UAS_r from *CIT2* upstream of a minimal *CYC1* TATA element driving a *lacZ* reporter cloned in the centromere-based *URA3* plasmid, pBL101, as previously described (32). Site-directed mutagenesis was used to change GGTCAC to GGTACC in the A element of the 76 bp UAS_r to produce p76XA′. A GTGACC-to-GGTACC change in the A′ element was made to construct p76AX′. Plasmid p76XX′ contains both sets of changes. p21A contains the sequence 5′-GCGAAGACCTCACGACCTATT-3′ with a single Rtg1p-Rtg3p binding site linked to the *CYC1* TATA. p76AA′, p76XA′, p76AX′, p76XX′, and p21A were transformed into COP161U7 cells and Δ*rtg3* [*rho*$^+$] and [*rho*^{0}] cells. Precultures of 15 to 20 pooled transformants were grown on selective medium to maintain the plasmids. Oligonucleotides used for site-directed mutagenesis are listed in Table 1. Precultures were used to inoculate YNBR cultures at a low density. Mid-logarithmic-phase cultures were harvested and assayed in triplicated for β-galactosidase activity as previously described (16). Plasmids were maintained at >95%.

RESULTS

Identification of *RTG3*. To identify new genes in the *CIT2* retrograde pathway, we took advantage of a strategy used previously to identify *RTG1* and *RTG2* (16). Briefly, the procedure involves the rescue by complementation with a wild-type yeast genomic library of mutants defective in expression of a *lacZ* reporter gene fused to the *CIT2* promoter. Because *CIT2* expression is greater in [*rho*^{0}] petite cells than in [*rho*$^+$] wild-type cells, the mutant screen and subsequent cloning experiments were carried out with a [*rho*^{0}] derivative of strain COP161U7. This strain was transformed with a centromere-based plasmid containing a 607-bp fragment of the 5′ noncoding region of *CIT2* fused to the *E. coli lacZ* gene. On 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates, colonies of this otherwise wild-type transformant stain blue. Cells were mutagenized with ethyl methanesulfonate to yield ~40% survivors and screened for colonies that were white on X-Gal plates. This screen yielded five independent mutants whose white-colony phenotype was not plasmid linked. The *CIT2-lacZ* reporter construct was then integrated into the *URA3* locus of each of the mutants. Following selection of the Ura$^-$ transformants on plates containing 5-fluoroorotic acid, these mutants were transformed with a YCp50 vector containing either *RTG1* (YCpRTG1) or *RTG2* (YCpRTG2) to determine whether any of the mutants were due to mutations in *RTG1* or *RTG2*. YCpRTG2 restored a blue-colony phenotype to four of the mutants, and none was restored to a blue-colony phenotype with YCpRTG1. Subsequent experiments revealed that the one mutant strain not rescued by either YCpRTG1 or YCpRTG2 was due to a single-gene mutation, which we have designated *rtg3-1*.

TABLE 1. Oligonucleotide sequences

Oligonucleotide No.[b]	Oligonucleotide Name	Sequence[a]	Use
1	5′-21-mer	CGAAGAGGTCACGACCTATTT	EMSA with 3′-21-mer
2	5′-1C	CGAAGCGGTCACGACCTATTT	EMSA with 3′-1C
3	5′-2T	CGAAGATGTCACGACCTATTT	EMSA with 3′-2T
4	5′-3T	CGAAGAGTTCACGACCTATTT	EMSA with 3′-3G
5	5′-4G	CGAAGAGGGCACGACCTATTT	EMSA with 3′-4G
6	5′-5A	CGAAGAGGTAACGACCTATTT	EMSA with 3′-5A
7	5′-6C	CGAAGAGGTCCCGACCTATTT	EMSA with 3′-6C
8	5′-7A	CGAAGAGGTCAAGACCTATTT	EMSA with 3′-7A
9	5′-8T	CGAAGAGGTCACTACCTATTT	EMSA with 3′-8T
10	5′-9C	CGAAGAGGTCACGCCCTATTT	EMSA with 3′-9C
11	5′-10A	CGAAGAGGTCACGAACTATTT	EMSA with 3′-10A
12	5′-23-mer	AAAAAAGTGTGACCTTTTCCGTA	EMSA with 3′-23-mer
13	5′-76 bp	gcattaggatcccCGGTGGTCATCGACTAGA	PCR of 76-bp fragment
14	3′-76 bp	gcattaggatccgCTACGGAAAAGGTCACA	PCR of 76-bp fragment
15	A-Kpn	AAATAGGTCGGTACCTGTTCGCCCC	Mutagenesis of A site
16	A′-Kpn	CCCTACGGAAAAGGTACCACTTTTTTTCTGC	Mutagenesis of A′ site

[a] Underlined residues indicate mutations of the wild-type core binding site, except for oligonucleotide 1, which is the wild-type site. Lowercase letters indicate non-*CIT2*. Sequences added for cloning purposes.
[b] Oligonucleotides 1 to 12 also exist as 3′ reverse complementary versions.

To clone the wild-type allele of *rtg3-1*, we transformed mutant cells with a YCp50-based wild-type yeast genomic library (30) and screened for colonies that were blue on X-Gal plates. From this screen, we obtained two colonies whose blue phenotype was plasmid linked; further analysis showed that the two restoring YCp50 plasmids contained overlapping inserts. The complementing function that could restore a blue-colony phenotype to the mutant strain was subcloned to a 4.2-kb insert and sequenced.

***RTG3* encodes a bHLH/Zip transcription factor.** DNA sequence analysis revealed a single open reading frame within the insert encoding a 486-amino-acid protein, hereafter called Rtg3p. The open reading frame corresponds to the region YBL103C (GenBank accession number U46012). Rtg3p contains sequence motifs characteristic of the bHLH/Zip family of transcription factors (Fig. 1) such as Max (3) and Mad (1). Unlike the case for Rtg1p, however, the basic domain of Rtg3p conforms closely to that of other bHLH proteins; in particular, it contains residues such as Arg_{287}, His_{290}, and Glu_{294} (Fig. 1B), which are highly conserved among bHLH proteins and which have been shown to make specific base or phosphate backbone contacts in the major groove of the canonical E-box binding site, CANNTG. Rtg3p also contains a relatively high proportion of Ser and Thr residues (19%), the majority of which are located in the amino-terminal half of the protein, before the bHLH motif (Fig. 1A). Finally, we have sequenced

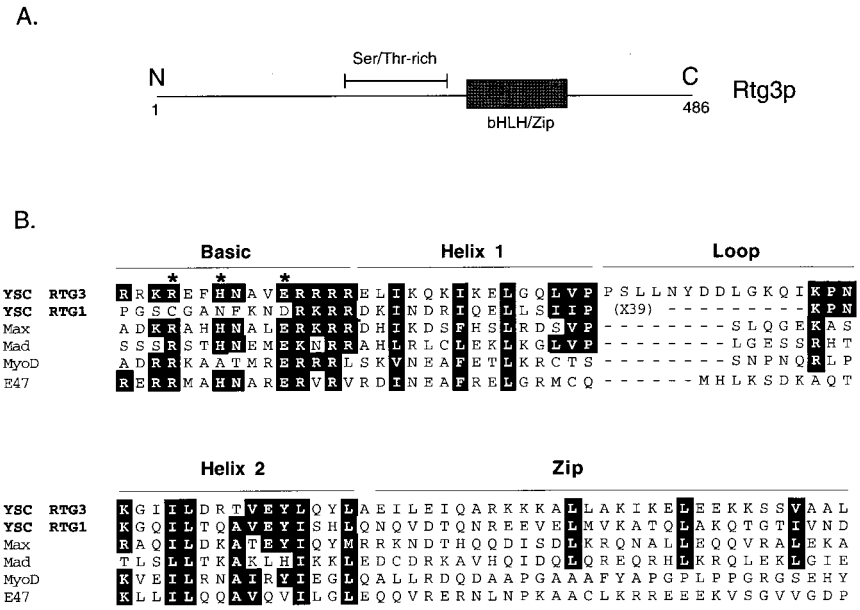

FIG. 1. (A) Diagrammatic representation of the 486-amino-acid Rtg3p showing the locations of the bHLH/Zip motif and the Ser/Thr-rich region. The *rtg3-1* allele has a C→T transition at position 955 resulting in a TAG stop codon at amino acid 153. (B) Amino acid alignment of the bHLH/Zip region of Rtg3p with other bHLH and bHLH/Zip proteins, including Rtg1p. The basic region, amphipathic helices I and II, the loop region, and the putative Zip region are indicated. The shaded areas indicate exact matches or conservative amino acid differences; asterisks indicate, from left to right, Arg_{287}, His_{290}, and Glu_{294} of Rtg3p.

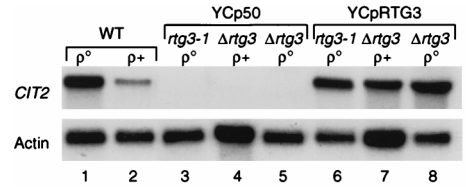

FIG. 2. *RTG3* is required for *CIT2* expression. Total cellular RNA was isolated from logarithmic-phase cultures of wild-type (WT) or *rtg3* mutant [*rho*$^+$] and [*rho*^{0}] derivatives of strain COP161U7 grown on YPR medium. Cells were transformed with a control YCp50 plasmid or a plasmid (YCpRTG3) containing the *RTG3* gene as indicated. The Northern blots were hybridized with probes specific for *CIT2* and actin mRNAs as described in Materials and Methods.

three independently cloned PCR products of the mutant *rtg3-1* allele and determined that the mutation is a C_{955}-to-T transition creating a UAG stop codon at amino acid position 153.

Rtg3p is required for *CIT2* expression. To assess the control of *RTG3* on *CIT2* expression, we performed Northern blot analysis of *CIT2* mRNA abundance in total RNA from [*rho*$^+$] and [*rho*^{0}] COP161U7 cells containing either a wild-type or a mutant allele of *RTG3*. In these experiments, we analyzed both the original *rtg3-1* mutant and a deletion mutant (Δ*rtg3*) in which codons 175 to 340 of the *RTG3* reading frame were replaced by the *URA3* gene. We note that Δ*rtg3* haploid cells were viable on YPD medium and that both the *rtg3-1* and Δ*rtg3* mutants could grow on glycerol and retain a [*rho*$^+$] mitochondrial genome during growth on fermentable carbon sources.

Figure 2, lanes 1 and 2, shows a typical retrograde response of the *CIT2* gene, where the abundance of *CIT2* mRNA is greater in [*rho*^{0}] than in [*rho*$^+$] cells. Lanes 3 to 5 show that *CIT2* mRNA is not detected in the original *rtg3-1* [*rho*^{0}] mutant or in [*rho*$^+$] or [*rho*^{0}] Δ*rtg3* cells transformed with the YCp50 vector alone. However, *CIT2* mRNA abundance is again restored to wild-type levels in both [*rho*$^+$] and [*rho*^{0}] cells transformed with YCpRTG3. After correction for the abundance of actin mRNA, the abundance of *CIT2* mRNA is four- to fivefold greater in the [*rho*^{0}] transformants than in the [*rho*$^+$] transformants. From these experiments, we conclude that *RTG3*, like *RTG1* and *RTG2*, is required for expression of the *CIT2* gene.

Growth requirements of Δ*rtg3* cells. Among the collateral phenotypes of cells with mutant alleles of *RTG1* or *RTG2* are the inability to utilize acetate as a sole carbon source and a growth requirement for glutamate or aspartate (16). To determine whether *rtg3* mutant cells have phenotypes similar to those of mutant *rtg1* and mutant *rtg2* cells, we compared the growth requirements of a Δ*rtg3* COP161U7 strain transformed with a YCp50 vector alone or with YCpRTG3. As controls, wild-type COP161U7 cells and an Δ*rtg1* derivative, transformed either with YCp50 vector alone or with YCpRTG1, were examined in the same experiment. Figure 3 shows that like *rtg1* mutant cells, Δ*rtg3* mutant cells (transformed with vector alone) were unable to grow on acetate medium and that they required either glutamate or aspartate for growth; these growth phenotypes were restored to wild type in Δ*rtg3* cells transformed with YCpRTG3.

Rtg1p and Rtg3p are together required for binding to the *CIT2* UAS$_r$. Previous studies showed that Rtg1p binds to the 76-bp *CIT2* UAS$_r$ element and requires, in addition to Rtg2p, at least one other protein for transactivation (32). The finding that Rtg3p, like Rtg1p, is a bHLH/Zip protein required for *CIT2* expression raises the possibility that Rtg3p is the partner protein to Rtg1p. Therefore, we first tested whether Rtg3p is required for Rtg1p-dependent complex formation with the 76-bp *CIT2* UAS$_r$, using extracts from wild-type and mutant *rtg1* and *rtg3* cells in EMSA. Figure 4A, lane 2, shows that extracts from wild-type cells yielded two prominent gel-retarded bands, A and B. Band B is the Rtg1p-specific complex, since it was not detected with extracts from Δ*rtg1* cells (lane 3). We also were unable to detect band B with extracts from either Δ*rtg3* or *rtg3-1* cells (lanes 4 and 5), indicating that Rtg3p also binds to the UAS$_r$ and is part of the same protein-DNA complex as Rtg1p.

The preceding observations suggest that Rtg1p and Rtg3p may interact as a heterodimer at their target site(s) in the *CIT2* UAS$_r$. To examine this possibility, we used purified recombinant Rtg1p and Rtg3p in a variety of EMSAs. Full-length recombinant Rtg1p and Rtg3p were expressed in *E. coli* as fusions to the C terminus of the *E. coli* maltose binding protein, MalE. The fusion proteins were purified from cell extracts and cleaved with factor Xa to separate the MalE protein from Rtg1p and Rtg3p. Figure 4B shows that neither Rtg1p or Rtg3p alone could band shift the 76-bp UAS$_r$ probe. However, when both proteins were present at roughly equimolar concentrations in the assay, a major gel-retarded band and two slower-migrating species appeared. Finally, as shown by the control in lane 4, no gel-retarded band was obtained in assays using the MalE protein by itself.

These data suggest that Rtg1p and Rtg3p interact as a heterodimer to bind to one or more sites in the *CIT2* UAS$_r$, since neither protein alone appears able to bind to any significant extent to that element. The observation that the mobility of the complexes formed with recombinant Rtg1p and Rtg3p (Fig. 4B) is greater than that of the Rtg1p-Rtg3p-dependent complex seen with extracts from yeast cells (Fig. 4A) suggests the possibility that one or more as yet unidentified proteins are recruited to the 76-bp UAS$_r$, perhaps by binding specifically to the heterodimeric complex. Indeed, evidence for such interactions involving bHLH proteins in other systems has been obtained. For example, the activation of muscle-specific genes has been suggested to occur via the formation of a ternary complex between the myocyte enhancer factor 2 and the MyoD-E12 bHLH heterodimer (22). And in yeast, transcriptional repression of the PHO4 bHLH transcription factor results from the masking of its activation by the repressor protein

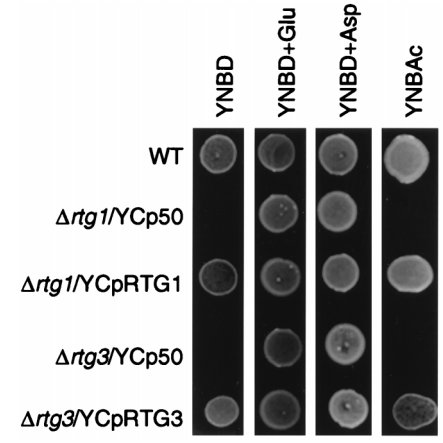

FIG. 3. Growth requirements of Δ*rtg3* and Δ*rtg1* cells. Δ*rtg1* and Δ*rtg3* COP161U7 strains were transformed with a control YCp50 plasmid and either YCpRTG1 or YCpRTG3. Cells from each of these cultures were spotted on YNBD, YNBD supplemented with 0.02% glutamate (YNBD + Glu) or 0.02% aspartate (YNBD + Asp), and YNBAc supplemented with 0.02% glutamate (YNBAc). The plates were incubated for 3 days at 30°C except for the YNBAc plates, which were incubated for 6 days at 30°C.

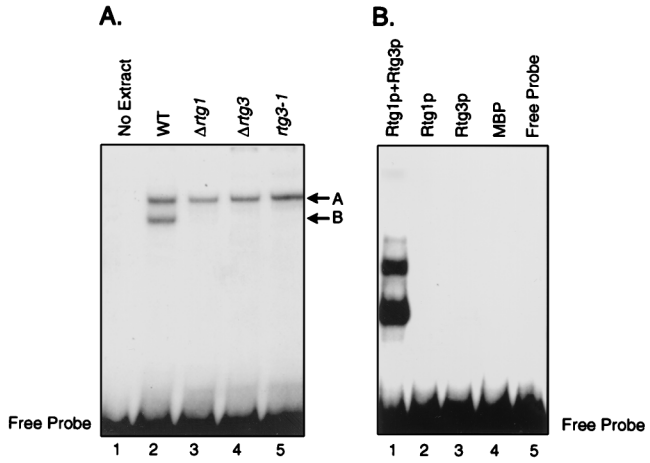

FIG. 4. Rtg1p and Rtg3p bind to the *CIT2* UAS_r. (A) Whole-cell extracts from wild-type (lane 2), Δ*rtg1* (lane 3), Δ*rtg3* (lane 4), and *rtg3-1* (lane 5) COP161U7 cells were prepared and tested in EMSA with the 76-bp UAS_r as described in Materials and Methods. Lane 1 is a no-extract control. (B) Purified, recombinant Rtg1p and Rtg3p were tested in EMSA with the 76-bp UAS_r. Lane 1, 5 ng of Rtg1p and 10 ng of Rtg3p; lane 2, 5 ng of Rtg1p; lane 3, 10 ng of Rtg3p; lane 4, 10 ng of maltose binding protein (MBP); lane 5, no added protein.

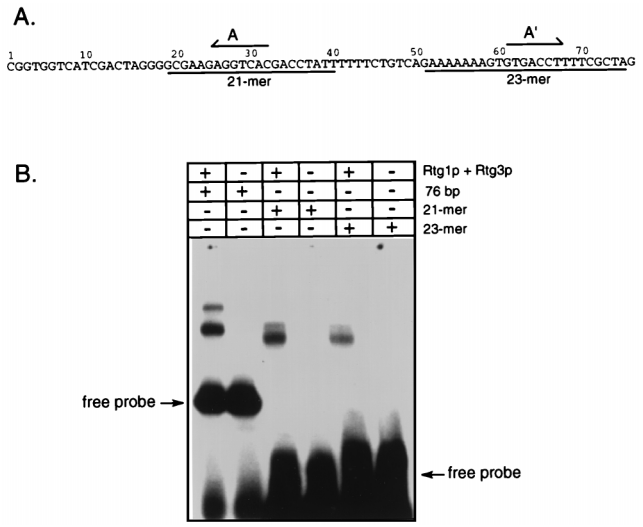

FIG. 5. (A) Sequence of the 76-bp UAS_r. The underlined sequences represent the 21-mer and 23-mer oligonucleotides used in EMSA spanning the A and A′ sites containing the inverted duplication of the sequence 5′-AGGTCAC-3′. (B) The double-stranded 21-mer and 23-mer and the 76-bp UAS_r were tested in EMSA for binding of Rtg1p and Rtg3p. Each reaction mixture contained either 5 ng of Rtg1p and 10 ng of Rtg3p or no added protein, as indicated.

PHO80, which binds to residues in PHO4 outside the bHLH domain (10). Further studies will be required to determine if other proteins—coactivators or repressors—interact with the Rtg1p-Rtg3p complex.

Rtg1p and Rtg3p bind to a non-E-box site. As already noted, the 76-bp *CIT2* UAS_r element does not contain a canonical bHLH E-box site. It does, however, contain a 7-bp inverted duplication of the sequence 5′-AGGTCAC-3′, designated A and A′ (Fig. 5A), with the E-box half site, CAC. To test whether the A and A′ sites might be involved in Rtg1p and Rtg3p binding, we made two double-stranded oligonucleotides, a 21-mer encompassing the A site and a 23-mer encompassing the A′ site (Fig. 5A). Each was then tested for the ability of recombinant Rtg1p and Rtg3p to bind in EMSA. Figure 5B shows that in the presence of Rtg1p and Rtg3p, both oligonucleotide probes gave rise to gel-retarded bands. As with the 76-bp probe, neither Rtg1p or Rtg3p alone bound to the oligonucleotide probes (not shown). These data suggest that Rtg1p and Rtg3p interact as a complex with at least two sites in the *CIT2* UAS_r encompassed by the oligonucleotides spanning the A and A′ sites.

To define more precisely the sequences to which the Rtg1p-Rtg3p complex binds, we carried out a series of EMSAs using recombinant proteins and 21-mers containing single-base transversion mutations of the A site as well as flanking base pair mutations. The results of these experiments (Fig. 6) show that single-base mutations of $(GTCAC)_{28–32}$ abolish binding completely: a G_{27}-to-T mutation reduced binding by about one-half, whereas various flanking mutations had no effect on binding. These experiments thus define a minimum sequence, 5′-GGTCAC-3′, necessary for binding of the Rtg1p-Rtg3p complex.

The Rtg1p-Rtg3p binding sites act synergistically in vivo. To establish an in vivo correlate to the sequence requirements for Rtg1p-Rtg3p binding in vitro, and to determine the contribution of each of the binding sites to the in vivo activity of the *CIT2* UAS_r, we made three constructs in which the A and A′ GGTCAC sites in the UAS_r were mutated singly and together to GGTACC. As shown in the preceding experiments, each of the single-base transversions blocks binding of the Rtg1p-Rtg3p complex (Fig. 6, lanes 6 and 7). Similarly, the Rtg1p-Rtg3p complex is unable to bind to a site with the double mutation (data not shown). To test for in vivo activity of the mutant UAS_r constructs, we made use of the centromere-based expression plasmid p76AA′, which contains the 76-bp UAS_r linked to a minimal *CYC1* TATA element driving the expression of a *lacZ* reporter gene. Plasmids containing the mutations of the A site (p76XA′), of the A′ site (p76AX′), and of both sites (p76XX′) were constructed. We also tested the in vivo activity of a single A binding site contained within just the 21-bp sequence used in the preceding EMSAs. Each of the constructs was then transformed into [*rho*$^+$] and [*rho*^{0}] COP161U7 cells, and β-galactosidase activity was measured in cell extracts. Figure 7 shows that, as previously described (16),

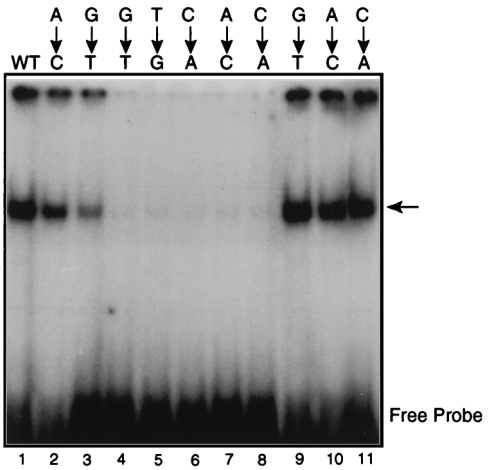

FIG. 6. Identification of the Rtg1p-Rtg3p binding site. Single-base transversion mutants of the double-stranded 21-mer containing the A site in the 76-bp UAS_r were tested in EMSA for the ability of recombinant Rtg1p-Rtg3p to bind. Reactions mixtures contained 5 ng of Rtg1p and 10 ng of Rtg3p. The arrow indicates the Rtg1p-Rtg3p complex.

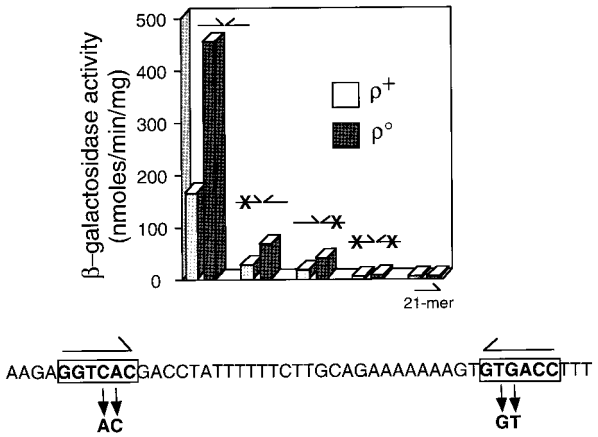

FIG. 7. In vivo activity of mutant Rtg1p-Rtg3p binding sites in the *CIT2* UAS_r. COP161U7 [*rho*$^+$] and [*rho*^{0}] cells were transformed with p76AA′, p76XA′, p76AX′, p76XX′, or p21A as described in Materials and Methods. Each of the plasmids contained the 76-bp UAS_r element or 21-mer used in the preceding experiments as indicated, linked to a minimal *CYC1* TATA element driving a *lacZ* reporter gene. The opposite-facing arrows indicate the A and A′ sites, and the X indicates a mutant site. Logarithmic-phase cultures grown on YNBR supplemented with Casamino Acids were harvested and tested for β-galactosidase specific activity.

the wild-type 76-bp UAS_r (p76AA′) not only supports *lacZ* expression of a reporter gene but does so in a [*rho*$^+$]-[*rho*^{0}]-responsive manner. Constructs containing mutations of the upstream site (p76XA′) result in about an 85% inhibition of *lacZ* expression in both [*rho*$^+$] and [*rho*^{0}] cells; mutations of the downstream site (p76AX′) result in a slightly greater (90 to 92%) inhibition of *lacZ* expression. When both sites are mutant (p76XX′), *lacZ* expression is inhibited nearly completely. The 21-bp construct containing just the A site and some flanking DNA sequences (Fig. 5A) was inactive. This result is not surprising, since DNA band shift experiments indicate that factors other than Rtg1p and Rtg3p are bound to sites within the UAS_r and are presumably required for *CIT2* expression. From these data, we conclude that both the A and A′ Rtg1p-Rtg3p binding sites are required for full [*rho*$^+$] and [*rho*^{0}] *CIT2* expression and that they act synergistically in transcriptional control.

***RTG3* expression in [*rho*$^+$] and [*rho*^{0}] cells.** Because *CIT2* transcription is absolutely dependent on Rtg1p and Rtg3p, it is possible that the difference in *CIT2* expression between [*rho*$^+$] and [*rho*^{0}] cells is due to a change in expression of *RTG1* and *RTG3*. However, in a previous analysis of Rtg1p (30), we could not detect any significant difference in the amount of the protein between [*rho*$^+$] and [*rho*^{0}] cells. We have carried out a similar analysis using rabbit polyclonal antibodies raised against recombinant Rtg3p. Although the anti-Rtg3p antiserum can readily detect the recombinant Rtg3p in Western blot analysis, we have been unsuccessful in detecting Rtg3p in extracts from either [*rho*$^+$] or [*rho*^{0}] cells (data not shown), suggesting that the abundance of the protein is very low. This view is consistent with the results of Northern blot analysis indicating that the *RTG3* mRNA abundance is also very low (data not shown). Therefore, we used a more sensitive RNase protection assay to compare the *RTG3* mRNA abundance in [*rho*$^+$] and [*rho*^{0}] cells. The results of such an experiment are presented in Fig. 8. Quantification of the protected fragments relative to actin show that *RTG3* mRNA is 2-fold greater in [*rho*^{0}] than in [*rho*$^+$] cells, whereas the *CIT2* mRNA abundance in the same RNA preparations is 6.6-fold greater in [*rho*^{0}] cells—a typical retrograde response for the *CIT2* gene. Similar results have been obtained in independent experiments of this sort. Clearly, there is not a one-to-one correspondence between *RTG3* and *CIT2* expression; however, this does not exclude the possibility that a small change in *RTG3* expression can be amplified to result in larger changes in *CIT2* expression between [*rho*$^+$] and [*rho*^{0}] cells. As noted in a previous study (15), we have not detected any difference in the EMSA pattern, either quantitatively or qualitatively, using the 76-bp element as a probe and extracts from [*rho*$^+$] or [*rho*^{0}] cells. However, these experiments do not rule out the possibility that mechanisms besides control of the abundance of Rtg1p or Rtg3p can affect the availability of these transcription factors for regulation of *CIT2* expression. Experiments are in progress to dissect the control of expression of both *RTG1* and *RTG3* and the possible relation between expression of these genes and the retrograde response.

DISCUSSION

Cellular requirements for *RTG3*. We have isolated and characterized a yeast gene, *RTG3*, required for basal and retrograde-regulated expression of the *CIT2* gene. Mutations of *RTG3* have multiple phenotypes, resulting not only in a loss of *CIT2* expression but also in an inability of the mutant cells to grow on acetate as a sole carbon source and in the appearance of a growth requirement for glutamate or aspartate (one amino acid will spare the other). These growth phenotypes are characteristic of cells with blocks in both the TCA and glyoxylate cycles (12). Although not presented in this study, mutant alleles of *RTG3* are also defective in the induction of peroxisomal proteins (and presumably peroxisome biogenesis) when cells are grown on medium containing oleic acid (3a). These phenotypes are identical to those observed in cells with mutant *rtg1* and *rtg2* alleles (4). Thus, we can classify *RTG3* as an additional member of the retrograde genes in yeast that function in a novel three-way interaction between mitochondria, peroxisomes, and the nucleus.

An Rtg1p-Rtg3p bHLH/Zip complex. The open reading frame of *RTG3* is predicted to encode a 54-kDa protein with a

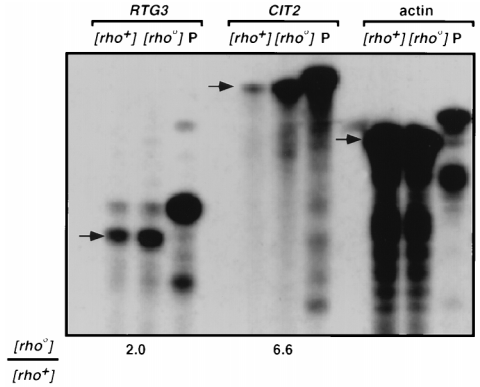

FIG. 8. RNase protection analysis of the abundance of *RTG3* and *CIT2* mRNAs in [*rho*$^+$] and [*rho*^{0}] cells. The levels of *RTG3* and *CIT2* mRNAs was carried out by RNase protection on total cellular RNA from wild-type COP161U7 [*rho*$^+$] and [*rho*^{0}] cells as described in Materials and Methods. For *CIT2* and *RTG3*, 50 μg of total RNA was used in the assay; for actin, 5 μg of RNA was used. P indicates the intact probes, which are 444, 323, and 196 nucleotides in length for *CIT2*, actin, and *RTG3*, respectively; the arrows show the RNase-protected fragments, which are 407, 282, and 176 nucleotides in length for *CIT2*, actin, and *RTG3*, respectively. The ratio of *CIT2* and *RTG3* mRNA abundance between [*rho*$^+$] and [*rho*^{0}] cells, normalized for actin mRNA abundance, is given at the bottom.

centrally situated bHLH/Zip motif that is very similar to the bHLH/Zip domains of transcription factors like Max (3, 7) and Mad (1). In addition, the amino-terminal half of Rtg3p upstream of the bHLH/Zip motif is very rich in serine and threonine residues, suggesting the potential for phosphorylation of the protein. bHLH/Zip transcription factors bind to their DNA target sites as homo- or heterodimers, utilizing dimerization interfaces that include the helix and zipper domains. We previously established that Rtg1p, also a bHLH/Zip protein required for basal and retrograde-regulated expression of *CIT2*, bound to one or more sites in the 76-bp *CIT2* UAS_r (32). Transactivation studies of reporter gene expression using fusions of the DNA binding domain of GAL4 to different domains of Rtg1p suggested that at least one other (unidentified) protein was required in together with Rtg1p for *CIT2* expression. The present study establishes that Rtg3p is the bHLH partner to Rtg1p for binding to the UAS_r. Using purified full-length recombinant Rtg1p and Rtg3p expressed in *E. coli*, we have shown that protein-DNA complex formation with the UAS_r requires both proteins, strongly suggesting that they interact with their DNA target sites as heterodimers or higher-order oligomers. In studies to be presented elsewhere, we have systematically analyzed the transactivation activities of various domains of Rtg3p in fusions to the DNA binding domain of GAL4. These analyses show a strong C-terminal activation domain and a weaker N-terminal activation domain of Rtg3p; neither of these domains, or the full-length Rtg3p, required either Rtg1p or Rtg2p for transactivation, suggesting that these proteins function, at least in part, to recruit Rtg3p to its binding site for transcriptional activation.

Detailed structural and crystallographic analyses show that the general mode of binding of bHLH proteins to their DNA target sites is through adjacent major groove contacts. These contacts are between specific amino acid residues in an extended α helix consisting of the basic region and helix 1 of each monomer to specific bases and phosphates within the E-box binding site (6, 7). These interactions thus position the α helices of each bHLH monomer unit perpendicular to each other on opposite sides of the DNA duplex. Contacts between monomer units are then established through coiled-coil interactions between the first helix of each subunit and the helix 2-Zip domains C-terminal to the loop regions. As elaborated in the following discussion, there are a number of reasons to believe that this general picture will be different for the interaction of the Rtg1p-Rtg3p complex with its target sites in the *CIT2* UAS_r.

The Rtg1p-Rtg3p binding site is novel. The E-box DNA recognition site for most bHLH proteins, CANNTG, may be either perfectly symmetric (but differ in bHLH binding specificity, such as CACGTG or CAGCTG) or lack perfect symmetry, such as CACCTG. In any case, a hallmark of the binding of bHLH/Zip proteins to these E boxes is the interaction of the invariant residues of an ERXR motif in the basic domain, as well as conserved His or Thr residues in the basic region, with the DNA (5). Crystal structure analysis shows, for example, invariant contacts between the conserved Glu of the ERXR motif and the CA of each E-box half site (6).

Our mutational analysis shows that the minimal binding site for Rtg1p-Rtg3p is 5′-GGTCAC-3′. The *CIT2* UAS_r contains two such sites arranged in opposite orientation, separated by 28 bp of an AT-rich inverted repeat; both sites bind the Rtg1p-Rtg3p complex and both are required for full expression of *CIT2* in vivo. The GGTCAC binding site, which we propose to call an R box, differs from the canonical bHLH E-box site in two obvious ways: it lacks dyad symmetry and it does not have a CA at its 5′ end. The location of the CAC at the 3′ end of the minimal hexanucleotide binding site poses interesting considerations of how the R box might be contacted by the Rtg1p-Rtg3p complex. Given the conserved Glu_{294}, Arg_{287}, and His_{290} residues in the basic region of Rtg3p that in other bHLH proteins contact the CA/GT of the E-box half site in the major groove, it is likely that the basic-helix 1 domain of Rtg3p is also positioned in the major groove, interacting with the CAC half site of the R box. The conserved Glu appears to be an important feature of the contact between the basic region of bHLH proteins and the CA of the symmetric E box because substitutions of that amino acid generally impair DNA binding (8, 9). Clearly, mutations of the CAC as well as the upstream GGT bases influence the binding of the Rtg1p-Rtg3p complex, whereas mutations of bases immediately downstream of the CAC have no effect (Fig. 6).

Two exceptions to the consensus bHLH–E-box binding are the SREB/ADD1 protein, which is involved in transcriptional regulation of the low-density lipoprotein receptor and in adipocyte differentiation (11, 39, 42), and the aryl hydrocarbon receptor (ARH)-aryl hydrocarbon receptor nuclear translator protein (ARNT) bHLH heterodimeric complex that functions in xenobiotic metabolism (27–29, 41). SREB/ADD1 is a bHLH protein that can recognize both E-box and non-E-box sites. In the latter case, a SREB/ADD1 homodimer binds to the site 5′-CACCCCAC-3′, which, although not an E box, contains a direct repeat of an E-box half site. A critical feature of the basic region of SREB/ADD1 that may accommodate binding to both E-box site and non-E-box sites is the substitution of a Tyr for the conserved Arg in the basic region of the protein (11). We have, in preliminary experiments, tested whether the recombinant Rtg1p-Rtg3p complex binds to E-box sites. For example, we have changed the R box to the E-box sequences, CAGCTG and CACGTG, while keeping the flanking DNA sequences the same. No significant binding was observed under conditions that yield strong binding to the wild-type R box (data not shown).

The Rtg1p-Rtg3p heterodimeric complex bears some similarities to AHR and ARNT. AHR and ARNT are bHLH proteins that form a heterodimeric complex that regulates transcription of genes encoding some enzymes of xenobiotic metabolism (27–29, 41). The basic region of AHR does not conform well to the basic region of most bHLH proteins, whereas the basic region of ARNT does. Moreover, the target binding sites for the AHR-ARNT complex are xenobiotic responsive elements in the 5′ flanking region of the responsive genes which are not consensus E boxes but rather have the consensus sequence 5′-T/GNGCGTGA/CG/CA-3′ (18). Thus, the similarities to the Rtg1p-Rtg3p complex include one member of a bHLH heterodimeric pair that has a nonconforming basic region and target sites that are not the consensus E box. The regulation of the AHR-ARNT system includes a specific ligand binding to AHR, which results in transcriptional activation of the target genes due to the translocation of these transcription factors from the cytoplasm to the nucleus. Our data also suggest the possibility that other proteins interact with or are recruited to the UAS_r complex by Rtg1p-Rtg3p. It will be interesting to learn whether any of the aforementioned properties can be extended to transcriptional activation by Rtg1p-Rtg3p.

We do not know what signal(s) activates *CIT2* transcription in cells with dysfunctional mitochondria. Although Rtg1p appears to be constitutively synthesized (32), it is possible that the relatively small change in expression of *RTG3* in [rho^0] cells triggers larger changes in *CIT2* expression. Alternatively, the retrograde response might be controlled by posttranslational modifications of these proteins such as phosphorylation-de-

phosphorylation, or alternative associations with regulatory subunits, as is the case for the negative regulator, Id (2) and mammalian homologs of the yeast transcriptional repressor, Sin3 (1, 35).

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