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The Regulation of *COQ5* Gene Expression by Energy Source

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The degree of severity of cardiomyopathy is inversely correlated with tissue levels of coenzyme Q (Q), suggesting that Q synthesis may impact the progression of the disease. It has been suggested that Q functions as an endogenously synthesized anti-oxidant, in addition to regenerating the potent anti-oxidants, vitamins E and C. However, very little is known about the mechanisms that regulate Q synthesis. Using the simple eukaryote *Saccharomyces cerevisiae* as a model, experiments have been designed to investigate the regulation of Q synthesis at the genetic level.

To investigate the regulation of *COQ5* gene expression by energy source, mRNA content was evaluated in yeast cells treated with dextrose, glycerol or oleic acid. After 1.5 h, more *COQ5* mRNA is produced by oleic acid treated cells than by glycerol treated. Experiments performed using *COQ5* promoter deletion/reporter constructs demonstrate a specific response to oleic acid. Additional promoter deletion analysis demonstrates that a non-fermentable carbon source element is also present, responding to both glycerol and oleic acid. The specific oleic acid response appears to be regulated by the Rtg family of transcription factors. This family of proteins is required for oleic acid-induced expression of genes of β-oxidation and peroxisomal proliferation, and plays an important role in co-ordinating mitochondrial/peroxisomal/nuclear communication in response to oleic acid, as well as defects in cellular respiration.

Keywords: Ubiquinone; Oleic acid; Gene expression

INTRODUCTION

Ubiquinone (coenzyme Q; Q) functions in the mitochondria to transport electrons between complexes I and III of the electron transport chain, and to accept electrons from succinate. In those organs that expend large amounts of energy, such as skeletal muscle and heart tissue, mitochondrial Q content is highly enriched.[1] One study found that heart tissue content of Q and the degree of severity of cardiomyopathy was inversely correlated.[2] However, the causality of the relationship between reduced levels of Q and increased severity of cardiomyopathy has not been established.

The ability of Q to alternate between reduced and oxidised states suggests that Q may act as an endogenously synthesized ant-oxidant.[3] However, the oxidative reactivity of Q is less than that of vitamin E, leading some to suggest that the biological role of Q is not as an anti-oxidant itself, but rather to regenerate vitamins E and C.[3] In non-mitochondrial locations, regeneration of Q appears to occur through plasma membrane-associated NADPH-Q reductase activity,[4] strengthening the argument that Q has a non-mitochondrial biological function. Given the role of Q in regenerating the anti-oxidant vitamins E and C, and its potential as an endogenous anti-oxidant, understanding the mechanisms regulating the synthesis of Q is critical.

The pathway of Q synthesis in *Saccharomyces cerevisiae* has been determined. Complementation analysis suggests that eight genes of Q synthesis exist in yeast,[5] and the gene encoding for each protein has been named (e.g. *COQ1*, *COQ2*, etc.). In yeast, expression of the *COQ* genes is believed to be regulated by carbon source, such that gene

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expression is repressed by dextrose and induced by non-fermentable carbon sources such as glycerol.[6] The expression of both *COQ3*[7] and *COQ7*[8] has been shown to be up-regulated by glycerol. However, specific transcriptional regulators of *COQ* genes have not been identified and promoter region mapping has not been accomplished.

The *COQ5* gene encodes for the protein 2-polyprenyl-6-methoxy-1,4-benzoquinone methyltransferase (Coq5p), which catalyses the C_3-methylation of the benzene ring midway through the synthesis pathway. The protein contains 307 amino acid residues, with a consensus mitochondrial targeting site from amino acids 21–28.[9] Consensus methylation signature sites also are present (amino acids 115–123; 137–141; 189–196; and 216–225.[10])

The 600 basepair (bp) promoter sequence of *COQ5* was analysed on the Internet using the SCPD promoter database (http://cgsigma.cshl.org/jian/). Consensus binding sites were identified for the transcription factors Gcr1 (general regulation of glycolytic genes), Mig1 (dextrose repression) and Rtg (an oleic acid responsive element which regulates mitochondrial/nuclear communication). The presence of these various carbon regulated binding sites in the *COQ5* promoter suggests that *COQ5* is a good candidate for investigating the regulation of Q synthesis by energy source.

The yeast Rtg family of transcription factors plays an important role in co-ordinating mitochondrial/peroxisomal/nuclear communication in response to oleic acid, or under conditions of reduced mitochondrial function, such as impaired respiration.[11] The family consists of three proteins, Rtg1p, Rtg2p, and Rtg3p. Rtg1p and Rtg3p are basic helix–loop–helix leucine zipper transcription factors which heterodimerize and bind to the R-box consensus sites (GTCAC) in the yeast promoter.[12] These transcription factors are essential for oleic acid-induced expression of some genes of β-oxidation, peroxisomal proliferation, and peroxisomal membrane proteins. However, only citrate synthase 2 has been shown to directly interact with these transcription factors.[12] Stimulatory conditions induce Rtg3p to migrate to the nucleus, complex with Rtg1p, and bind DNA to induce gene transcription. The localisation of Rtg3p is believed to be regulated by its phosphorylation state because cytosolic Rtg3p is hyperphosphorylated while nuclear Rtg3p is partially dephosphorylated. The phosphorylation state of Rtg3p is regulated by Rtg2p in response to unidentified cellular signalling mechanisms.[11]

The present work investigates the regulation of *COQ5* gene expression by different energy sources, dextrose, glycerol, and oleic acid. The results of this study suggest that *COQ5* gene expression is differentially upregulated by glycerol and oleate, and that the oleate response is controlled by the Rtg family of transcription factors.

TABLE I Genotypes of yeast strains used in this study

Strain	Genotype	Source
SEY6210	MATα *ura3 leu2 lys2 his3 trp1 suc2*	P. Trotter
PSY142pt	MATα *ura3 leu2 lys2*	R. Butow
PSY142.1	MATα *ura3 leu2 lys2 rtg1Δ::LEU2*	R. Butow
PSY142.3	MATα *ura3 leu2 lys2 rtg3Δ::LEU2*	R. Butow

METHODS

Yeast Strains and Growth

Yeast strains (Table I) were maintained in rich medium.[13] For growth on alternative carbon sources, synthetic media (YN: 6% yeast nitrogen base, 0.5% yeast extract, 1% tergitol NP-40, and amino acids) supplemented with 2% dextrose (YNDex), 3% glycerol (YNGly) or 1.0 mM oleate (YNO) were used.

Coenzyme Q_6 Extraction and Analysis

The yeast form of coenzyme Q, Q_6, was extracted from yeast grown to saturation in dextrose, diluted back to early log phase ($OD_{600} = 0.3$) and inoculated in YNDex, YNGly, or YNO. Samples were taken at time points, pelleted and dried under nitrogen. Coenzyme Q_6 was extracted as published.[14] In brief, pellet weight was determined and the pellet resuspended in water. A known amount of Q_9 was added to the suspension as internal standard, 0.5 mm glass beads added and cells were lysed by vortexing. Lipids were extracted with methanol: petroleum ether, to achieve a final proportion of 0.5:6:4 (water:methanol:petroleum ether, v:v). Phases were separated by centrifugation and the petroleum ether layer reserved. The samples were analysed for Q_6 and Q_9 by HPLC on a Zorbax ODS column (Sigma) and absorbance at 275 nm monitored. The amount of Q_9 recovered was calculated and used to correct for lipid losses during extraction. Data are expressed as μg Q_6/g dry weight of cells.

Regulation of *COQ5* mRNA Expression by Energy Source

After pre-growth on YNDex, cells were washed and grown in either YNO or YNGly medium at 30°C. At time points, aliquots of cells were removed and total RNA isolated using Tri-Reagent (Molecular Research Center) following manufacturer's instructions. At one hour, an aliquot was removed, 2% dextrose added and cells grown an additional 30 min. Following standard techniques,[15] equal amounts

of total RNA were fractionated, transferred to membranes, and probed for *COQ5* mRNA using ^{32}P labelled DNA probes generated in our laboratory by PCR. Membranes also were probed for the yeast small nucleolar RNA, *U3*, to confirm that equal amounts of RNA were present in each lane.

Promoter Deletion/reporter Constructs

To investigate whether the *COQ5* promoter responds differently to various carbon sources, promoter deletion/reporter constructs were designed. Two lengths of promoter DNA were generated by PCR using high fidelity Expand Polymerase (Boehringer–Mannheim) and cloned in-frame into the yeast lacZ vector YEp357 (ATCC). The full length 600 bp promoter was amplified with a forward primer annealing at 600 bp upstream of the *COQ5* start site, and a reverse primer annealing across the start site. A truncated 400 bp fragment was generated which is missing the Rtg and the Mig consensus sequences by using a forward primer annealing at 400 bp upstream and the reverse primer across the start site. All constructs were sequenced at the DNA Sequencing Core Facility (The University of Texas at Austin) and compared to the published promoter sequence to confirm that PCR induced mutations were not present in the DNA product. Wild-type yeast were transformed with the promoter/lacZ constructs using the Yeastmaker Transformation Kit (Clontech). To induce β-galactosidase activity, cells were grown overnight in 2% dextrose defined medium, followed by 8 h in 0.5% dextrose. Promoter activity was measured after an additional 18 h in 2% dextrose, 3% glycerol, or 0.2% oleic acid defined media.

Following treatments, β-galactosidase activity was assayed as described.[16] In brief, cells were solubilized in 50 mM sodium phosphate buffer, pH = 7.0, containing 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol. The solubilized pellet was re-extracted and centrifuged at 11,500 *g* for 5 min and the supernatant reserved as soluble cell protein. Protein concentration was determined by the BCA protein assay (Pierce) following manufacturer's instructions. The crude extract was assayed for β-galactosidase activity in extraction buffer using ONPG (*O*-nitrophenyl-β-D galactoside; Sigma) as substrate. Colour change was monitored spectrophotometrically at 420 nm and data expressed as nmol ONPG converted/mg/min.

RESULTS

Modification of Q_6 Content by Carbon Source

To establish that the Q content of yeast increases with growth on non-fermentable carbon sources, yeast were grown on various media, Q_6 extracted, and Q_6 content determined by HPLC. The results (Fig. 1) show that both oleic acid and glycerol induce Q_6 synthesis in yeast, although the time course of the response is slower in glycerol than oleate. Induction of Q synthesis after growth in either glycerol or oleate was expected, because both media are non-fermentable carbon sources for *Saccharomyces cerevisiae.*

Regulation of *COQ5* Gene Expression by Carbon Source

Computer analysis of the promoter regions of the yeast genes for Q synthesis revealed one gene containing a known oleic acid responsive element (the Rtg family).[12] This gene, *COQ5*, encoding for a C-methyltransferase, also contains a consensus binding site for Mig1 (a dextrose repression element).[6] To determine whether these sites were functional, northern analysis of samples collected after growth on oleic acid or glycerol was performed (Fig. 2). After one hour of treatment, there is a similar level of expression of *COQ5* mRNA in the dextrose control and the glycerol treated groups, and slightly more *COQ5* mRNA present in oleate treated cells. This difference in *COQ5* mRNA levels between oleate and glycerol treated groups is more marked at 1.5 h. After an additional 30 min of dextrose, *COQ5* mRNA is not present in either treatment group. This response to dextrose is typical of carbon source regulated genes in yeast and probably is due to both dextrose repression of gene transcription through the Mig1 site, and to dextrose induced mRNA degradation.[6] These results suggest that both the Mig1 and the Rtg consensus binding sites are active in the *COQ5* promoter. In addition, the data show that *COQ5* mRNA is induced to a greater degree by oleic acid than by glycerol.

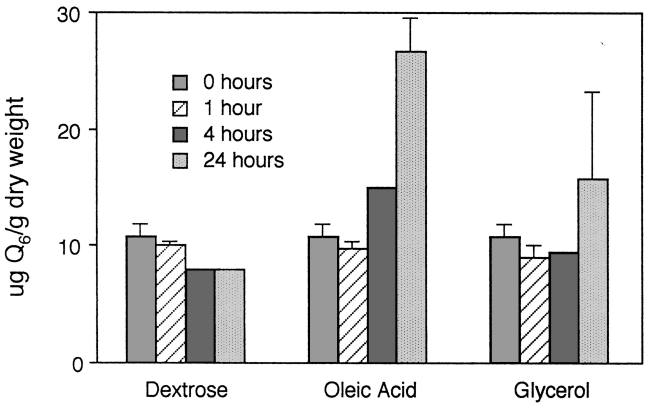

FIGURE 1 The CoQ_6 content of yeast fed non-fermentable carbon increases with time. Wild-type yeast was grown for 24 h in defined medium containing dextrose, glycerol, or oleic acid as carbon source. Coenzyme Q_6 was extracted, and analysed by HPLC.

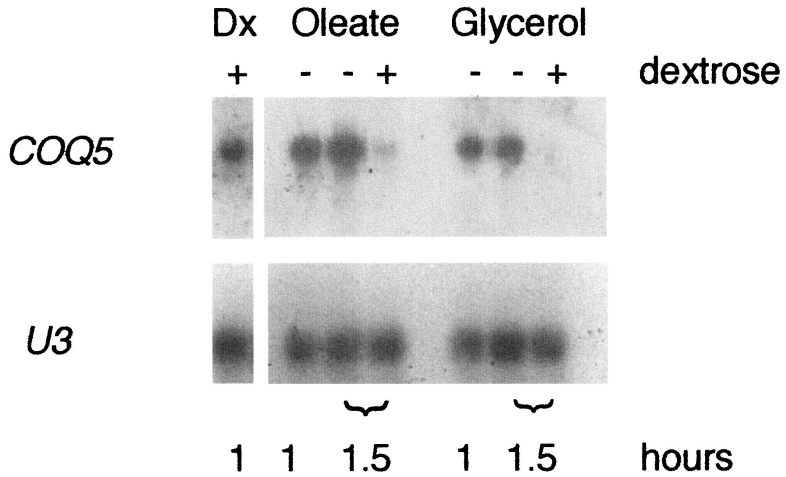

FIGURE 2 *COQ5* mRNA is increased by oleic acid treatment. Wild-type yeast was pre-grown in dextrose followed by 1 and 1.5 h of treatment with glycerol or oleic acid. At 1 h, some samples had dextrose added back. Total RNA was extracted and expression of *COQ5* analysed. The membrane also was probed for the small nucleolar RNA, *U3*, to ensure that equal amounts of total RNA were present in each lane.

Regulation of the *COQ5* Promoter by Oleic Acid

To demonstrate that the induction of *COQ5* mRNA by oleic acid is due to increased gene transcription and not to differential mRNA decay rates on oleic acid versus glycerol, promoter deletion/reporter constructs were generated. The full-length (600 bp) construct was compared to a 400 bp construct, truncated to remove the Rtg and the Mig1 consensus binding sites. The data (Fig. 3) show that the complete *COQ5* promoter responds more strongly to oleic acid than to glycerol, compared to the basal level of activity found upon dextrose. The truncated promoter grown on dextrose shows a higher basal level of activity, probably due to the loss of the Mig1 dextrose repression element. Both oleic acid and glycerol induce the truncated construct more strongly than does dextrose. The strong response to these non-fermentable carbon sources suggests that an unidentified non-fermentable carbon source element may be present in this region. These data confirm that oleic acid induces the complete *COQ5* promoter to a greater extent than either glycerol or dextrose.

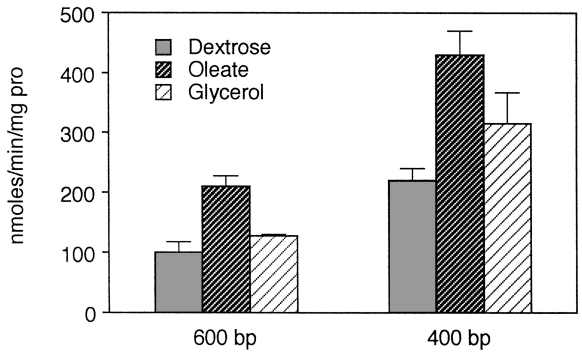

FIGURE 3 The entire *COQ5* promoter responds to oleic acid, while the truncated promoter responds to non-fermentable carbon. Yeast strains containing the *COQ5* promoter cloned in-frame to lacZ were treated with dextrose, glycerol or oleic acid for 18 h. Extracts were prepared and β-galactosidase activity measured.

Role of Rtg3p in the Response of *COQ5* to Oleic Acid

To determine whether the Rtg element plays a role in the control of *COQ5* gene expression, experiments were designed to examine the induction of the full-length reporter construct by oleic acid in strains (Table I) that were deleted in either Rtg1p or Rtg3p (kindly provided by R. Butow; University of Texas, Southwestern Medical School). In these experiments, β-galactosidase activity will not be induced by oleic acid if the deleted transcription factor is required for the oleic acid induced response of the promoter.

In Fig. 4, the strain deleted in Rtg1p expresses levels of β-galactosidase activity which are similar to the wild-type strain after treatment with oleic acid. Therefore, Rtg1p is not required for the oleic acid-induced response of the *COQ5* promoter. However, levels of activity in the Rtg3p deletion strain after oleic acid treatment are similar to basal activity on dextrose. Thus, Rtg3p appears to be required for the oleic acid induced response of the full-length *COQ5* promoter. The literature predicts that the response of the Rtg1p and Rtg3p deletion strains will be different. In the absence of Rtg1p, some Rtg3p is found in the nucleus. However, in the absence of Rtg3p, Rtg1p remains localized in the cytosol and DNA binding does not occur.[11] Thus, our results support the hypothesis that the oleic acid induction of *COQ5* gene expression is regulated by the Rtg transcription factor family, specifically by Rtg3p activation.

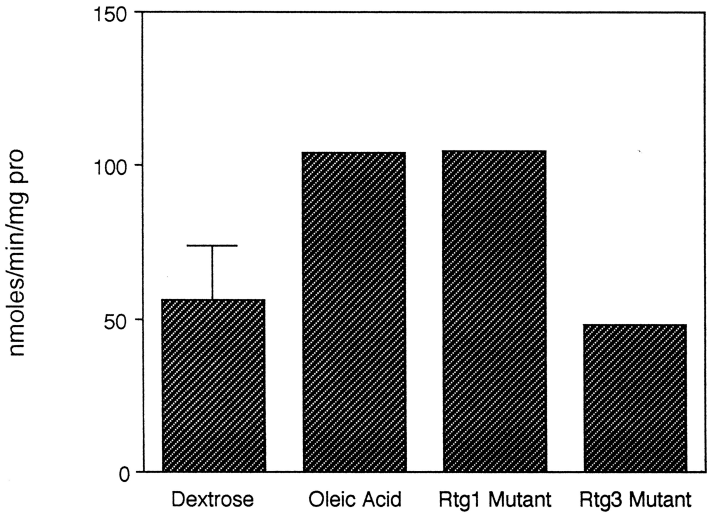

FIGURE 4 The *COQ5* promoter is not induced by oleic acid in a strain deleted in *Rtg3*. Wild-type yeast strains were treated with dextrose (column 1) or oleic acid (column 2) for 18 h. Strains deleted in Rtg1p (column 3) or Rtg3p (column 4) were transformed with the *COQ5* promoter-lacZ construct and treated with oleic acid for 18 h. Extracts were prepared and β-galactosidase activity measured.

DISCUSSION

In summary, analysis of the yeast *COQ5* promoter indicated that *COQ5* gene expression might be regulated by energy source. Our experiments confirm that the *COQ5* gene contains a functional dextrose repression element, Mig1. The factors responsible for the regulation of the *COQ5* promoter by non-fermentable carbon sources have not been identified, but our data suggest that a non-fermentable carbon response element occurs in the 400 bp upstream region of the promoter. The induction of *COQ5* mRNA expression by oleic acid appears to be through the Rtg transcription factor family. Another oleic acid responsive transcription factor family exists in yeast. This family consists of two proteins, Oaf1p and Pip2p, which heterodimerize in response to oleic acid treatment and induce the transcription of various genes of β-oxidation and other peroxisomal proteins.[17] Although the consensus binding site for this family is not found in the *COQ5* promoter sequence, it has been suggested that a second, unidentified response element may exist for this family.[17] The possibility that the response of the *COQ5* promoter to oleic acid is regulated by this second family of transcription factors was eliminated by results showing that strains deleted in Oaf1p or Pip2p displayed similar levels of *COQ5* promoter activity after treatment with oleic add when compared to a wild-type strain (data not shown). Therefore, this second family of oleate-responsive transcription factors does not appear to regulate the oleic acid response of the *COQ5* promoter, as was predicted by computerised promoter analysis.

In conclusion, a gene of coenzyme Q synthesis is differentially regulated by energy source. The expression of the gene *COQ5* is regulated by the Rtg family of transcription factors. This family mediates communication between the mitochondria and the nucleus in response to oleic acid, and under conditions where respiration is impaired.

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