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RESEARCH ARTICLE

Lin Lu · George Roberts · Kirk Simon · Jia Yu
Alan P. Hudson

Rsf1p, a protein required for respiratory growth of *Saccharomyces cerevisiae*

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Abstract A central problem in our understanding of mitochondrial (mt) function remains the question of how coordinate transcriptional control is accomplished between nucleus and mitochondria. Here, we report the initial characterization of a protein of previously unknown function, the product of the YMR030 W gene, that appears to mediate such coordinate gene expression. Expression of YMR030 W is glucose-repressible; a deletion mutant for this gene shows a severe growth defect on glycerol-, but not glucose- or ethanol-based medium. In that mutant, transcript levels from *GUT1* and *GUT2* are highly attenuated compared with those of the wild-type parent when both are grown on glycerol-based medium. Under the same growth conditions, transcripts from the mt *OLI1* gene, which has one copy of a mt upstream activating sequence (UAS) in its 5′-flanking region, are attenuated in the ΔYMR030 W mutant, but mRNA from the mt *COX3* (*OXI2*) gene, which lacks the mt UAS, are not. Some nuclear genes encoding mt-related proteins also show low transcript levels in the ΔYMR030 W mutant in comparison with those of the wild-type parent strain during glycerol-based growth. Localization of the protein, via its expression fused to green fluorescent protein, indicates that it is present in both nucleus and mitochondria, supporting a respiration-related transcriptional role for this gene product in both cellular genetic compartments. Because of its role in both respiratory growth and mt function, we designate the YMR030 W coding sequence *RSF1* (respiration factor 1).

Keywords Respiration · Mitochondria
Transcriptional control · Mitochondrial gene expression

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L. Lu · G. Roberts · A.P. Hudson (✉)
Department of Immunology and Microbiology,
Wayne State University School of Medicine,
Gordon H. Scott Hall,
540 East Canfield Avenue,
Detroit, MI 48201, USA
E-mail: ahudson@med.wayne.edu
Tel.: +1-313-9936641
Fax: +1-313-5771155

K. Simon
Department of Biology,
Eastern Michigan University,
Ypsilanti, Michigan, USA

J. Yu
Department of Medicine,
University of Pennsylvania School of Medicine,
Philadelphia, Pennsylvania, USA

A.P. Hudson
Medical Research Service,
Department of Veterans Affairs Medical Center,
Detroit, Michigan, USA

Introduction

Normal respiratory growth of the yeast *Saccharomyces cerevisiae* requires properly controlled expression of many genes encoded on both the nuclear and mitochondrial (mt) genomes. Any discussion of the means by which such complex genetic activity is accomplished must include three related issues. The first concerns mechanisms that individually and coordinately govern expression of mt-related and other relevant nuclear genes. The second deals with mechanism(s) controlling transcription of mt genes within the organelle, and the third comprises the means by which expression of functionally related genes from each genome might be coordinately regulated. We have a good understanding of the mechanisms governing the expression of mt-related and other nuclear genes, and recent studies have given some insight into how nucleus and mitochondria interact. However, our knowledge of the mechanisms by which transcription of mt genes is controlled remains relatively poor.

A long-term interest of this laboratory is the control of mt gene expression in yeast. Our previous work indicated that, under certain growth conditions, tran-

scription of most yeast mt genes is cAMP-sensitive and that the mechanism responsible for that control requires regulated activity of a cAMP-dependent protein kinase (PKA; McEntee et al. 1993; Rahman et al. 1994; 1995a, 1995b). Details of the mechanism responsible for cAMP-mediated regulation of mt transcript initiation in yeast remain to be elucidated, but some observations strongly suggest that this organellar transcriptional control system operates via a *trans*-activation process formally similar to those described for nuclear and vertebrate mt systems (McEntee et al. 1993). Such a mechanism would involve a sequence-specific, PKA-dependent protein–DNA interaction for transcriptional initiation. In an earlier study, we identified a DNA sequence element on the yeast mt genome which appears to function in such a system, but the protein(s) interacting with that element are unknown (Iqbal et al. 1996). This mt upstream activating sequence (UAS) is a GC-rich element present 5′ to seven of the 12 or so strong promoters on the organellar genome, and it is located at varying distances and in both orientations in relation to those promoters.

We are also interested in whether and, if so, how expression of functionally related nuclear and mt genes is governed in concert in yeast. One means by which such coordinate nuclear–mt transcription might be accomplished is via use of the same *cis*- and/or *trans*-acting elements upstream of related genes in both genomes. We performed a computer-based search comparing the putative mt UAS with all DNA sequences in the yeast nuclear genome, and that analysis showed that the element is not present there. The analysis further indicated that the sequence of the element is not closely related to that of any known *cis*-acting DNA element in yeast or other eukaryotes. Nonetheless, the *HAP1* gene product governs expression of *CYC1* and *CYC7* via binding to two UASs of somewhat different DNA sequence (Pfeifer et al. 1987). Thus, even though the mt element is not held in common among functionally related nuclear and mt genes in yeast, it is conceivable that a common *trans*-activator might interact with UAS elements of different DNA sequence to coordinately activate gene expression in both genetic compartments.

In the work described here, we demonstrate that the putative mt UAS can function in transcriptional activation in the yeast nucleus, even though it is not naturally present there. Using that mt sequence element in a one-hybrid screening procedure, we identify a nuclear coding sequence of previously unknown function, YMR030 W, specifying a protein that apparently interacts with the mt UAS in the nucleus. We demonstrate that the product of this gene is required for growth on glycerol as carbon source via its control of *GUT1* and *GUT2* expression. Further, we present results indicating that the YMR030 W gene product is present in both nucleus and mitochondria and that its functions include both control of mt gene expression and transcriptional governance of some mt- and respiration-related nuclear genes. We therefore designate the YMR030 W coding sequence *RSF1* (for respiration factor 1).

Materials and methods

Yeast strains and cell growth

The yeast strains used in these experiments were: W303-1A (*MAT*α leu2 his3 trp1 ura3 ade2 ϱ^+), Y294 (*MAT*α leu2 trp1 ura3 his3 ϱ^+; a gift from J.R. Broach), BY4741 (*MAT*α his3 leu2 met15 ura3 ϱ^+; ATCC), ΔYMR030 W (*MAT*α his3 leu2 met15 ura3 ΔYMR030 W ϱ^+; ATCC), and W303/*rsf1* (*MAT*α leu2 his3 trp1 ura3 ade2 YMR030 W::URA3 ϱ^+; prepared in this laboratory, see: Yeast plasmids, cloning, insertional inactivation, and transformations). Prior to experiments, all strains were carried on rich medium with 2% glucose as sole carbon source. For experiments, minimal medium with appropriate supplements was used, with 2% glycerol, 2% ethanol, or 2% glucose as sole carbon source (Sherman 1991). Cell growth for all experiments was at 30 °C in a temperature-controlled dry-shaker orbiting at 260 rpm. The growth rate for specific strains in some experiments was monitored by recording the absorbance at 600 nm (A_{600}).

Preparation of nucleic acids

For all experiments, cells were harvested by centrifugation during early log-phase growth ($A_{600} = 0.7$) and total nucleic acids were prepared from washed cell pellets via homogenization in hot phenol, as described by McEntee and Hudson (1989). DNA was isolated from the total nucleic acid preparations by extensive treatment with RNaseA and RNase T1 (Life Technologies, Bethesda, Md.), followed by phenol:chloroform extraction and collection via ethanol precipitation. Pure RNA was prepared by treatment of total nucleic acid preparations with DNaseI (RQ1; Promega Life Sciences, Madison, Wis.), followed by phenol:chloroform extraction and collection via ethanol precipitation. RNA preparations were assessed for residual DNA by PCR, using primers targeting the *ACT1* gene, in the absence of reverse transcription (RT). cDNA was prepared for standard and real-time RT-PCR analyses using the MuLV enzyme (Life Technologies) and random hexamers as primers, as described by Iqbal et al. (1996).

Yeast plasmids, cloning, insertional inactivation, and transformations

For some studies, the shuttle vector used was pBM947 (a generous gift from Dr. L.W. Bergman). This plasmid is ca. 9 kbp in length, contains the *CENIV* and *ARS1* sequences, and uses *URA3* as a selectable marker. In addition, the vector includes the *HIS3*-coding sequence under the control of the *GAL1* TATAA element, and 5′ to that sequence is an *Eco*RI-cloning site in which potential *cis*-regulatory DNA sequences can be inserted. For some studies, we cloned various nuclear and mt DNA sequences of roughly equal length into that site, transformed these plasmids into the *his3* strains W303-1A and Y294 (Ito et al. 1983), and then assessed the growth rate of transformants in histidine-lacking minimal medium containing either a respiratory or fermentative carbon source. The GC-rich mt UAS element used in this clone was included in a 114-bp restriction fragment from the region immediately 5′ to the mt *OLI1* gene, described previously (Iqbal et al. 1996). Irrelevant sequences of about the same length and of known AT + GC content were prepared by PCR from the yeast nuclear and mt genomes and then cloned into the *Eco*RI site 5′ to *HIS3* in pBM947. A deletion mutant for the YMR030 W coding sequence (ΔYMR030 W) and its wild-type parent strain (BY4741) were obtained from the ATCC (see above: Yeast strains and cell growth). We also prepared our own mutant for this coding sequence by standard insertional inactivation of the YMR030 W sequence in wild-type strain W303-1A, using a

construct prepared by PCR which included a *URA3* cassette. The primers used to make that construct were: 5′-GAT AAGTAACAGTCTAGA*GATGCTAAGAGATAGTGATG*-3′ and 5′-GATCAGTTACATTCTAGA*GTCCTGTCTTATTGTTCTTG*-3′ (bases *in italics* indicate the upstream- and downstream-most sequences of the *URA3* cassette). This PCR product was restricted with *Xba1* and subcloned into *Xba1* sites in the insert of a bacterial plasmid that spanned nucleotides +233 to +1,100 of the YMR030 W coding sequence; the construct was linearized by restriction, transformed into strain W303-1A, and its proper insertion into the YMR030 W sequence on chromosome XIII was confirmed by PCR. Most experiments described here were done in both the ATCC and W303-1A strains, to be sure that results were not dependent on a specific nuclear background. For complementation of the mutants, a PCR product was prepared from yeast genomic DNA (strain W303-1A) that included the YMR030 W coding sequence plus approximately 700 bp of the 5′-flanking and 300 bp of the 3′-flanking sequences. The primers used to produce the PCR product were: 5′-ATTATAGGATCCCAATTCTT GCTTTCATTT-3′ and 5′-ATTATTGGATCCTGATTACTTTT GCTTATGAA-3′. The PCR product was cloned into the pRS316 vector (a generous gift from Dr. N. Davis), the resulting plasmid (pRS/*RSF1*) was transformed into the mutant for YMR030 W in each nuclear background, and mutants were then assessed for growth under respiratory and fermentative growth conditions.

One-hybrid analysis

To identify proteins interacting with the mt sequence element in the yeast nucleus, the MATCHMAKER™ one-hybrid system (Clontech, Palo Alto, Calif.) was employed, in concert with a high-quality yeast genomic DNA library kindly supplied by Dr. P. James in the proper pGAD vector (James et al. 1996). A triple copy of the UAS was produced by PCR, this was used to construct the double-reporter strain required in the analysis, and identification of clones surviving double selection and all other aspects of the analysis were done according to manufacturer′s instructions. The inserts of clones so identified were subjected to DNA sequencing at the core facility at Wayne State University. The two coding sequences represented by those clone inserts, YMR030 W and YJR127C, were identified by searching the complete yeast genome sequence. In this publication, we describe functional studies only for the product of the YMR030 W coding sequence. Functional characterization of the YJR127C gene product will be presented in another context.

Real-time PCR and RT-PCR analyses

SYBR green-based real-time PCR analysis was used to assess mt genome copy number in some experiments, and similarly structured real-time RT-PCR assays were employed to assess various transcript levels in other experiments. These analyses were done as described by us and others (e.g., Hiratsuka et al. 1999; Gérard et al. 2001; Wang et al. 2001); the sequences targeted and primers employed for these studies are given in Table 1. Each assay was repeated several times, with each tube run in triplicate each time, and signals from these analyses were normalized to values obtained for the 18S rRNA gene (PCR) or its transcript (RT-PCR/cDNA) run simultaneously in triplicate with the experimental tubes. All analyses were done in a model 7700 sequence detector (PE Biosystems, Foster City, Calif.) and data were analyzed using Sequence detection software ver 1.7 (PE Biosystems).

Analysis of cellular location of the YMR030 W gene product

The full coding sequence of YMR030 W was produced by PCR using a high-fidelity DNA polymerase and cloned into the *CEN/ARS* vector pUG35 (generously supplied by Dr. J.H. Hegemann). This vector is approximately 6,200 bp in length, uses *URA3* as a selectable marker for transformation, includes the *MET25*-inducible promoter, and includes the yEGFP3 coding sequence in the polylinker region. The YMR030 W sequence was inserted in-frame immediately N-terminal to the yEGFP3 sequence and the construct was transformed into the wild-type strain BY4741. Empty plasmid was transformed in parallel as a control. Transformants were fixed in 3.7% formaldehyde for 30–60 min and then stained with 100 ng/ml 4′,6-diamidino-2-phenylindole (DAPI)/ml (Sigma, St. Louis, Mo.) for 60 min in the dark. Transformants so treated were examined using a Nikon Eclipse™ E600 microscope with a DAPI filter/fluorescein-isothiocyanate setting and photographed using a digital camera.

Table 1 Genes targeted and primer sequences used in real-time PCR and/or RT-PCR analyses

Gene	Primer
Mitochondrial genes	
21S rRNA	5′-CCGTAATGTAGACCGACTCAG-3′
	5′-TGGAGCAGAGTTCACACCTTA-3′
OLI1	5′-GCAATTAGTATTAGCAGC-3′
	5′-ACAGAATAAACCTGTAGC-3′
COX3 (*OXI2*)	5′-AATGCATGGTTATATTGGTAAT-3′
	5′-AGCTTCAGCTACAATATCTCTAA-3′
Nuclear genes	
YMR030 W	5′-ACTCCAAGGATACTTAATATGAA-3′
	5′-TGAGTAACGTTGACAGGAGAT-3′
RPO41	5′-GATCGTTGGTGGACTACTGCTG-3′
	5′-ACCACCCAAGGCAGCATAAT-3′
MIP1	5′-AACGAGGGACAAGTATGACG-3′
	5′-GAGAACCATCGAGCACAAAA-3′
COX4	5′-GAAACTTTGATTGGTCCTGGTGCTAA-3′
	5′-GGAGAACCCGTACAACCGACATAAC-3′
MRP13	5′-CATTACAACCGAGACCTC-3′
	5′-ATGTACGTCCAAATAGCG-3′
ACT1	5′-ACTATTGGTAACGAAAGATTCA-3′
	5′-ACACTTCATGATGGAGTTGTAA-3′
18S rRNA	5′-CGGCTACCACATCCAAGGAA-3′
	5′-GCTGGAATTACCGCGGCT-3′

Results

The GC-rich mt DNA sequence can activate transcription in the nucleus

One copy of the GC-rich mt UAS was cloned 5′ to *HIS3* in the vector pBM947 and the construct was transformed into the *his3* strain W303-1A. Growth of those transformants was assessed in histidine-lacking minimal medium with glycerol or glucose (see below, in this section) as carbon source. As shown in Fig. 1A, cells given empty vector showed no growth over 120 h in respiratory medium. In contrast, cells transformed with the vector containing one copy of the mt UAS grew in this medium, although at a rate slower than that of the same *his3* strain with empty plasmid in histidine-supplemented medium. Repeats of the experiments using different transformants carrying empty vector and vector including the mt element gave results identical to those shown, as did experiments using transformants of the *his3* strain Y294 (data not shown). PCR was used to produce a DNA fragment containing three tandem copies of the mt UAS;

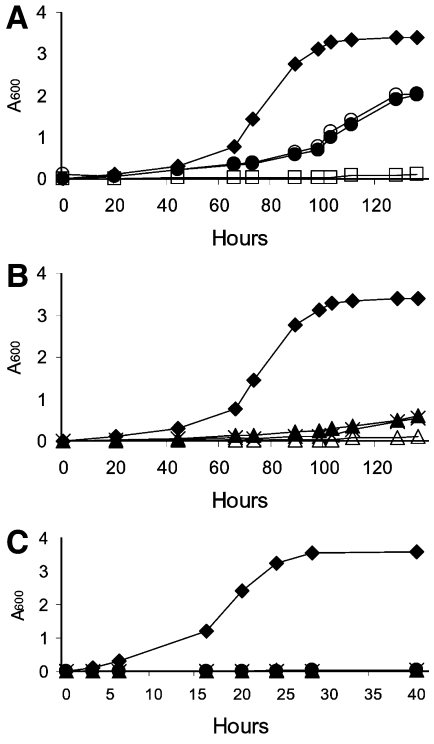


Fig. 1A–C Growth curves for strain W303-1A transformed with plasmid pBM947 containing various insert sequences and using minimal medium lacking added histidine with either 2% glycerol (**A**, **B**) or 2% glucose (**C**) as carbon source. Plasmids were constructed as described in Materials and methods and growth was at 30 °C. Symbols in each panel indicate cells carrying pBM947 with the following inserts: *black diamonds* no insert but with added histidine in the medium, *white squares* no insert and no added histidine in the medium, *black circles* one copy of the mitochondrial (mt) *cis*-element, *white circles* three copies of the mt *cis*-element, *black triangles* a nuclear DNA fragment with 72% AT content, *crosses* a mt DNA fragment with 75% GC content, *white triangles* a nuclear DNA fragment with about equal AT and GC content. The growth curves were also repeated with appropriate transformants of wild-type strain Y294 and identical results were obtained

and that fragment was cloned into pBM947. The growth rate of W303-1A transformants in glycerol-based, histidine-lacking medium was identical for cells carrying either the 3× or 1× mt UAS element (Fig. 1A).

To assess whether expression of *HIS3* in these experiments depended specifically on the presence of the mt UAS, irrelevant DNA fragments of ca. 150 bp length were cloned into pBM947, the constructs transformed into strain W303-1A, and growth studies repeated. As shown in Fig. 1B, a fragment containing 75% GC from the yeast mt genome did not permit growth in histidine-lacking, glycerol-based medium; the sequence of this GC-rich fragment was unrelated to that of the mt UAS. Similarly, neither a fragment of about equal GC + AT content from the nucleus, nor a nuclear DNA fragment with 72% AT activated expression of *HIS3* when cloned 5′ to that gene. Growth of all W303-1A transformants was also assessed in minimal medium with glucose as carbon source. As shown in Fig. 1C, neither plasmids containing the single- or the triple-copy mt UAS, nor those plasmids carrying irrelevant sequences allowed growth on this carbon source without added histidine. Thus, the mt UAS is able to activate expression of an associated gene in the nucleus, but it appears to do so only under respiratory growth conditions.

Identification of proteins interacting with the mt DNA sequence

A commercial system for one-hybrid analysis was used to identify the protein(s) interacting with the mt element to allow transcription of *HIS3*. Transformants were selected on glycerol- rather than glucose-based medium, since data given above suggested that the protein(s) at issue might not be expressed at a high level under fermentative growth conditions. Two independent screenings of approximately 1.5×10^7 plasmids each identified about 28 plasmids that repeatably allowed survival through the double *HIS3*/*lacZ* selection. Plasmid DNA was prepared from each surviving transformant and the sequence of each insert was determined. Of the 28 plasmids, 15 contained identical or overlapping insert sequences from YMR030 W, an ORF on chromosome XIII specifying a protein of unknown function. Three plasmids contained identical or overlapping insert sequences from YJR127C, an ORF on chromosome X encoding a second protein of unknown function. The remaining plasmids each contained a different insert and their growth was the weakest of all transformants that survived the selection. We considered these to be false positives and discarded them. In this report, we describe studies relating to the YMR030 W gene product; studies on the YJR127C gene product will be presented separately.

Expression of YMR030 W on respiratory vs fermentative growth medium

Standard RT-PCR was done to confirm that the YMR030 W coding sequence is actually expressed in wild-type cells, and these experiments indicated that transcripts from the ORF were present in cells grown under both respiratory and fermentative conditions (data not shown). However, visual assessment of the final amplicon intensity on ethidium bromide-stained gels indicated far lower levels of RNA from this coding sequence in glucose- than in glycerol-grown cells. To determine the relative expression level of YMR030 W, quantitative real-time RT-PCR was used to define transcript levels from that coding sequence in wild-type strain W303-1A growing on glycerol- and ethanol- vs glucose-based medium. Fig. 2 presents the results of such analyses, which indicated that expression of YMR030 W is subject to glucose repression. This is consistent with data above suggesting that respiratory growth conditions are necessary for high-level expression of the coding sequence and/or interaction of its gene

Fig. 2 Real-time RT-PCR assays targeting relative transcript levels from the YMR030 W coding sequence in wild-type strain W303-1A during respiratory vs fermentative growth. Cells were grown and total RNA/cDNA was prepared as described in Materials and methods. Real-time RT-PCR analyses were done as given in Materials and methods, using primers listed in Table 1. Relative transcript levels were normalized to 18S rRNA and indexed to the value obtained for strain W303-1A grown in glucose-based medium. Data shown are from three independent experiments in which each tube was run in triplicate. *Bars* indicate standard error

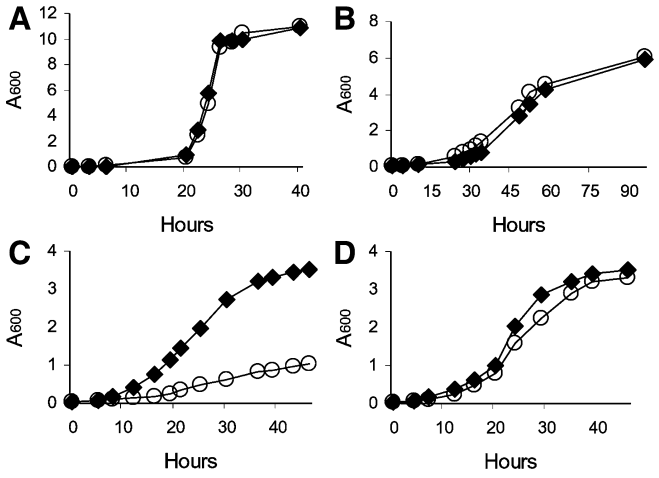


Fig. 3 Representative growth curves for wild-type strain BY4741 (*black diamonds*) and the mutant ΔYMR030 W (*white circles*) in glucose-based (**A**), ethanol-based (**B**), and glycerol-based (**C**, **D**) medium. **D** The ΔYMR030 W mutant had been transformed with a plasmid whose insert included the entire YMR030 W coding sequence plus several hundred base pairs of the 5′- and 3′-flanking sequences

product with the mt UAS. The data in Fig. 2 further show that, while transcripts from YMR030 W were induced strongly during growth in glycerol-based medium, they were induced to a lesser extent in ethanol-based medium, suggesting that the encoded protein is involved importantly in glycerol-based respiratory growth.

The YMR030 W gene product is required for growth in glycerol-based medium

We reasoned that, if the YMR030 W gene product functions importantly in glycerol-based respiratory growth, then a mutant for that gene should grow poorly in medium containing glycerol as sole carbon source. We obtained the deletion mutant for YMR030 W (ΔYMR030 W) and its otherwise isogenic wild-type parent strain (BY4741) from the ATCC; we also prepared a mutant (W303/*rsf1*) for this coding sequence by insertional inactivation in a different nuclear background. The growth curves in Fig. 3 demonstrate that the mutant for YMR030 W in the BY4741 background doubled at the same rate as did its parent strain in glucose-based (Fig. 3A) and ethanol-based medium (Fig. 3B). However, growth of this mutant was strongly attenuated in glycerol-based medium, compared with that of its wild-type parent (Fig. 3C); that attenuated growth rate in glycerol-based medium was restored nearly to normal when a plasmid containing the YMR030 W coding sequence, plus several hundred base pairs of 5′- and 3′-flanking sequences, was introduced into the mutant (Fig. 3D). Repeats of these growth and complementation experiments using the mutant for YMR030 W in the W303-1A background gave results identical to those in Fig. 3, confirming these observations are not strain-dependent (data not shown). Thus, the YMR030 W gene product is required for respiratory growth when glycerol is the sole carbon source, but it is less/not critical for growth in ethanol-based medium (but see: The YMR030 W gene product influences mt transcription).

Transcript levels from *GUT1* and *GUT2* are attenuated in the ΔYMR030 W mutant

Studies from other laboratories showed that growth of yeast on medium containing glycerol as sole carbon source requires expression of the genes *GUT1* and *GUT2*, which encode the enzymes glycerol kinase and glycerol-3-phosphate dehydrogenase, respectively (e.g., Pavlik et al. 1993; Rønnow and Kielland-Brandt 1993). Because our observations indicated that the YMR030 W gene product is important for glycerol-based growth of yeast, we assessed the expression of *GUT1* and *GUT2* in the ΔYMR030 W mutant (BY4741 background), using a quantitative real-time RT-PCR assay system. The results of these analyses, given in Fig. 4, clearly showed that transcript levels from both *GUT1* (Fig. 4A) and *GUT2* (Fig. 4B) were severely attenuated in the mutant, although mRNA from each gene is induced to a small extent, thereby explaining the less than total growth deficit of the mutant in medium containing glycerol as sole carbon source.

The YMR030 W gene product influences mt transcription

Because we identified the YMR030 W coding sequence via its apparent interaction with the mt UAS, we asked

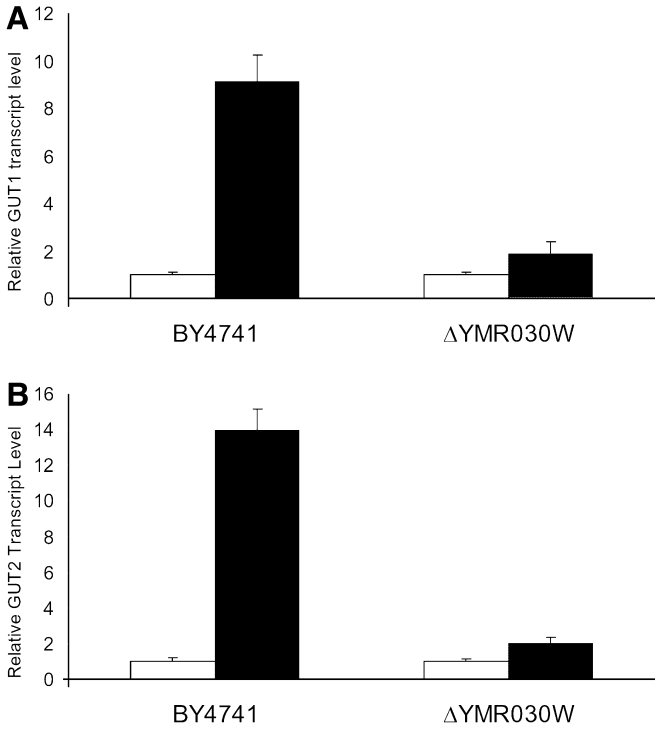


Fig. 4 Real-time RT-PCR assays targeting relative transcript levels from the *GUT1* (**A**) and *GUT2* (**B**) genes in strains BY4741 and ΔYMR030 W grown in glycerol- vs glucose-based medium. Cells of the wild-type and mutant strains were grown and total RNA/cDNA was prepared as described in Materials and methods. The real-time RT-PCR analyses were done as given in Materials and methods, using primers listed in Table 1. Data were normalized to levels of 18S rRNA in these analyses and relative transcript levels indexed to that obtained for each strain in glucose-based medium. Data shown are from three independent experiments in which each tube was run in triplicate. *Bars* indicate standard error. *White columns* Glucose-based medium, *black columns* glycerol-based medium

whether organellar transcript levels were affected in the ΔYMR030 W mutant during glycerol- and ethanol-based respiratory growth. Real-time RT-PCR analyses showed that messenger levels from the mt *OLI1* gene, which has one copy of the GC-rich mt UAS in its 5′-flanking sequence, were severely attenuated in the mutant, compared with those in the parent strain during growth in glycerol-based medium (Fig. 5A). Interestingly, they were also attenuated during growth in ethanol-based medium (Fig. 5B), although the level of that attenuation apparently was not significant enough to engender a noticeable growth defect in the mutant during growth in ethanol-based medium (see Fig. 3B). We obtained similar results to those for the *OLI1* gene in experiments targeting the mt 21S rRNA gene, which has two copies of the mt element in its upstream region (data not shown). In contrast, transcripts from the mt *COX3* (*OXI2*) gene, which lacks the mt UAS in its upstream region, were not affected in the glycerol-grown ΔYMR030 W mutant strain (Fig. 5C) or when that mutant was grown in ethanol-based medium (data not shown). Importantly, the attenuated levels of *OLI1* mRNA and the normal levels of *COX3* mRNA in the glycerol-grown ΔYMR030 W mutant were produced from wild-type levels of mt DNA (Fig. 5D). These results suggest that the product of the YMR030 W gene is involved in mt transcriptional control specifically for those organellar genes that possess the mt UAS in their upstream control.

The YMR030 W gene product influences expression of some mt-related nuclear genes

GUT1 and *GUT2* are both nuclear genes. Gut1p, the kinase, is a cytoplasmic (extra-mt) enzyme, but Gut2p, the dehydrogenase, is located within mitochondria. Because the YMR030 W gene product influences transcript levels from at least some mt genes as well as the mt-related nuclear gene *GUT2*, we asked whether other nuclear genes encoding mt products could also be affected in the ΔYMR030 W mutant. Real-time RT-PCR was used to assess relative transcript levels from several nuclear genes specifying such products in that mutant during glycerol-based respiratory growth. These experiments showed that transcripts from *RPO41*, the gene encoding the catalytic subunit of the mt RNA polymerase, and *MIP1*, which specifies the single-subunit mt DNA polymerase, were not affected by loss of the YMR030 W gene product (Fig. 6A, B). However, mRNA levels from *COX4*, which encodes subunit IV of cytochrome oxidase, and *MRP13*, which specifies a mitoribosomal protein, were both significantly lower in the ΔYMR030 W mutant than in its wild-type parent (Fig. 6C, D); essentially identical results were obtained when these assays were repeated using RNA/cDNA from the ΔYMR030 W mutant in the W303-1A background (data not shown). Thus, the YMR030 W gene product does influence the expression of some, but not all, nuclear genes encoding products directly required for mt function.

Cellular localization of the YMR030 W gene product

The transcriptional effects exerted on *GUT1*, *GUT2* and other nuclear genes by the YMR030 W gene product suggest that this protein functions within the nucleus. While the protein may be present within mitochondria, the similar effects on expression identified for the mt UAS-associated organellar genes do not necessarily require the YMR030 W protein to be located within the organelle. That is, the mt transcriptional effect observed in the ΔYMR030 W mutant for *OLI1* and the 21S rRNA gene may be a downstream event mediated by some nuclear gene product whose expression is attenuated by loss of the YMR030 W protein. To assess whether the YMR030 W gene product is actually present only in the nucleus or in both nucleus and mitochondria, the full YMR030 W coding sequence was cloned in the pUG35 plasmid in-frame just upstream of

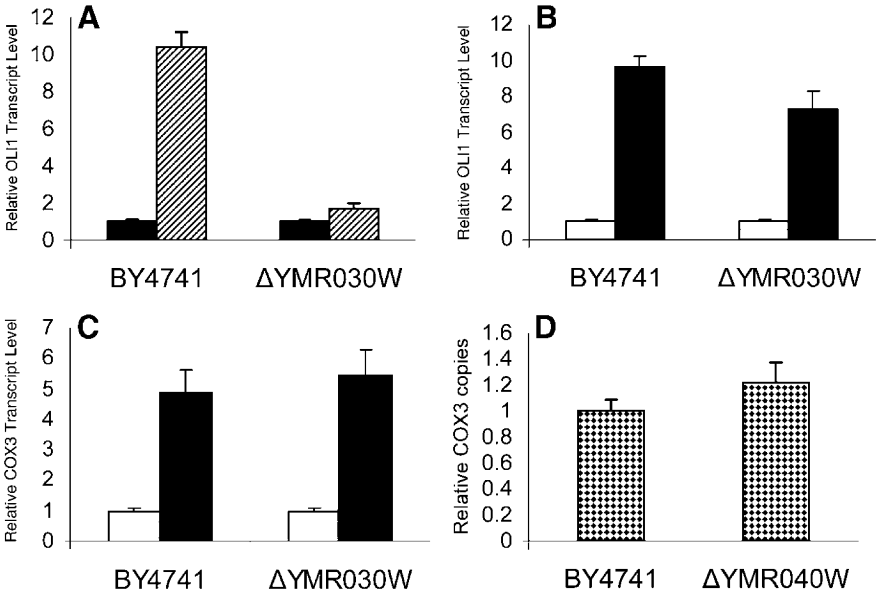
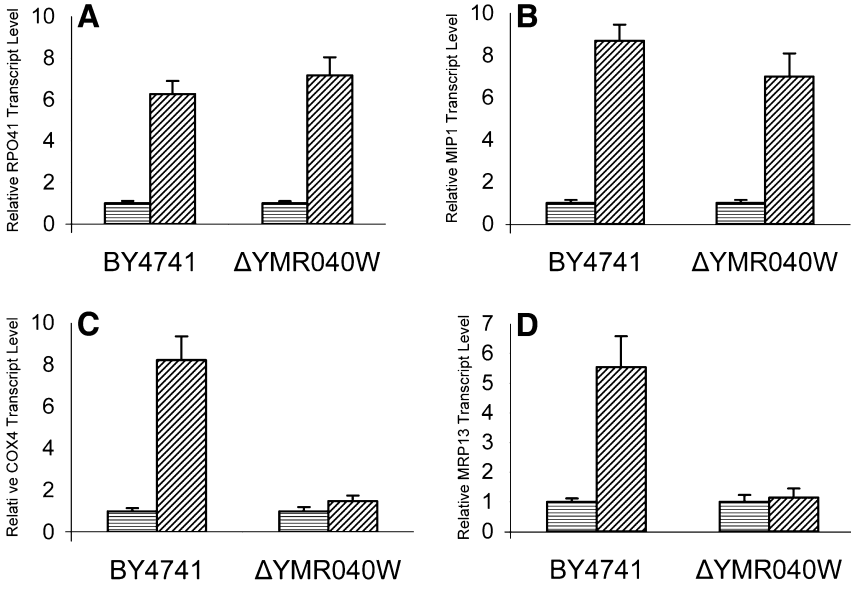


Fig. 5 Real-time RT-PCR assays targeting relative transcript levels from the mt *OLI1* gene (**A** glycerol- vs glucose-based medium, **B** ethanol- vs glucose-based medium), which has an associated GC-rich mt upstream activating sequence (UAS), and the *COX3* (*OXI2*) gene (**C** glycerol- vs glucose-based medium), which has no associated mt UAS, in strains BY4741 and ΔYMR030 W. **D** Real-time PCR analysis to define relative levels of the mt *COX3* (*OXI2*) gene in the wild-type parent (BY4741) and ΔYMR030 W mutant strains, indicating that the level of mt DNA in the mutant and parent strains do not differ. Cells of the wild-type and mutant strains were grown and total RNA/cDNA or DNA was prepared as described in Materials and methods. The real-time RT-PCR and PCR analyses were done as given in Materials and methods, using primers listed in Table 1. In **A**, **B**, and **C**, data were normalized to the levels of 18S rRNA and relative transcript levels indexed to the value for each strain in glucose-based medium. *Left columns* Glucose-based medium, *right columns* glycerol- or ethanol-based medium. In **D**, data were normalized to the levels of 18S rDNA in the total DNA preparation and the level of mt DNA in the mutant indexed to that of the parent strain. Data shown are from three independent experiments in which each tube was run in triplicate. *Bars* indicate standard error

a sequence specifying the green fluorescent protein (GFP). When expressed in yeast cells transformed with this construct, a chimeric protein is produced consisting of the YMR030 W gene product with GFP fused to its C-terminus. The construct was introduced into the wild-type strain BY4741 and the transformants were stained

Fig. 6 Real-time RT-PCR assays targeting relative mRNA levels from the mt-related nuclear genes *RPO41* (**A**), *MIP1* (**B**), *COX4* (**C**), and *MRP13* (**D**) in strains BY4741 and ΔYMR030 W grown in glycerol- vs glucose-based medium. Cells of each strain were grown and total RNA/cDNA was prepared as described in Materials and methods. The real-time RT-PCR analyses were done as given in Materials and methods, using primers listed in Table 1. Data were normalized to the levels of 18S rRNA in these analyses and relative transcript levels indexed to that obtained for each strain in glucose-based medium. Data shown are from three independent experiments in which each tube was run in triplicate. *Bars* indicate standard error. *Horizontal hatching* Glucose-based medium, *diagonal hatching* glycerol-based medium



with DAPI in order to co-localize the GFP signal with that of nuclear and mt DNA. The results of these experiments are presented in Fig. 7. Control transformations using an empty pUG35 plasmid showed no specific subcellular localization, as expected (Fig. 7A); and DAPI staining of those transformants highlighted both nuclear and mt DNA, again as expected (Fig. 7B). The plasmid containing the complete YMR030 W coding sequence fused to GFP yielded a protein product that was clearly identifiable in the yeast nucleus (Fig. 7C) and the data further suggested that it was present in mitochondria (Fig. 7D). We also asked whether the YMR030W–GFP fusion protein would complement the glycerol-specific growth defect of the deletion mutant. In these experiments, expression of the fusion protein in the ΔYMR030 W strain growing in glycerol-based medium raised the growth rate by about 10% over that of the mutant lacking the fusion construct (data not shown). This limited complementation is presumably due to improper folding of the YMR030 W gene product with its GFP tail, since that tail is approximately the same size as the protein to which it is attached. Thus, the product of the YMR030 W gene appears to be a transcriptional modulator operating in both cellular genetic compartments.

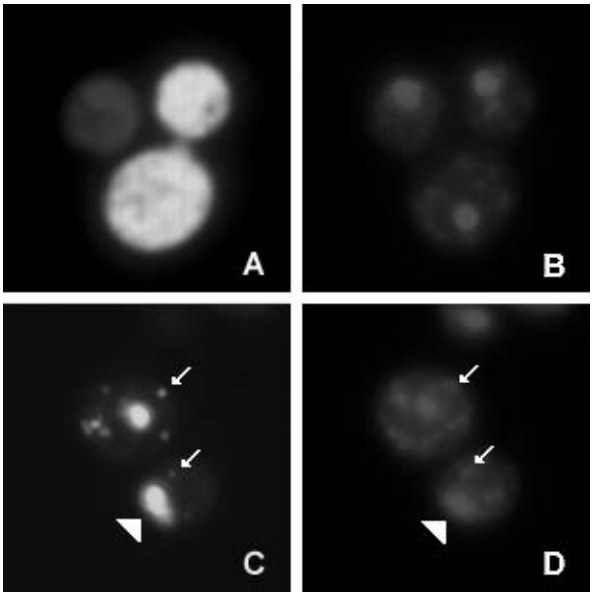


Fig. 7A–D Photomicrographs of strain BY4741 transformed with empty plasmid pUG35, or that plasmid containing the full YMR030 W coding sequence cloned in-frame immediately upstream of the yEGFP3 (GFP: green fluorescent protein) sequence and stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were grown on glucose-based medium and prepared for visualization as described in Materials and methods. **A** Cells given empty pUG35 and visualized for GFP, **B** cells given empty pUG35 and visualized for DAPI, **C** cells given pUG35 with the YMR030W-GFP construct and visualized for GFP, **D** cells given pUG35 with the YMR030W-GFP construct and visualized for DAPI. Magnification is 600× in all panels. *Arrowheads* indicate nuclear location. *Arrows* indicate mt location

Discussion

This laboratory is interested in the mechanism(s) governing transcript initiation on the yeast mt genome and the means by which coordinate nuclear–mt transcriptional control might be accomplished. In the present work, we identified the YMR030 W coding sequence on the basis of the apparent interaction of its gene product with a GC-rich mt UAS element, which we showed previously to be involved in cAMP-dependent organellar gene expression (Iqbal et al. 1996). Interestingly, that apparent protein–mt DNA interaction occurred in the nucleus to activate transcription of *HIS3* on a plasmid. It is possible that our identification of YMR030 W was a fortuitous result of this unusual experiment. Nonetheless, the data presented here demonstrate that a mutant for this coding sequence shows a growth deficit on glycerol-based medium and that transcripts from some mt and some mt-related nuclear genes not involved in glycerol utilization are affected significantly by loss of the YMR030 W gene product. Thus, the product of this gene is required for respiratory growth of yeast under at least some growth conditions. Importantly, the data presented here further suggest this protein may function as a mediator of coordinate transcriptional control for respiration-related genes in both the nuclear and mt genomes. We therefore designate the YMR030 W coding sequence as *RSF1* (respiration factor 1).

We do not fully understand as yet the precise function(s) the *RSF1* gene product plays in respiration via its effects on nuclear and mt gene expression. Rsf1p is a protein of 376 amino acids and initial computer-based (PSORTII; http://psort.nibb.ac.jp) analysis of its structure indicated no obvious DNA-binding motifs, *trans*-membrane regions, canonical PKA phosphorylation sites, or standard mt transit sequences. Such sequences are not absolutely required for organellar access, and data presented here strongly suggest that Rsf1p is present within mitochondria. The computer-assisted analysis did identify six apparent nuclear localization signals, consistent with our demonstration that Rsf1p is present in the yeast nucleus, and it identified a possible signal sequence cleavage site at amino acids 39–40 (Fig. 8). A BLAST search of the Rsf1p amino acid sequence through available protein databases indicated some homology to a region of the human and mouse *SOX-6* transcription factors and to a region of the *β′* subunit of RNA polymerase I. Given its effects on expression of the several nuclear genes examined here, Rsf1p probably is a transcription factor, even though it lacks an easily identifiable DNA-binding motif (see also below).

From our experimental results, we do not know whether Rsf1p operates as a *trans*-activator directly on DNA sequences in the upstream flanking region of *GUT1*, *GUT2*, or the other nuclear genes examined here whose expression is modulated by loss of this gene product. Transcriptional control for the two genes encoding products required for glycerol metabolism has

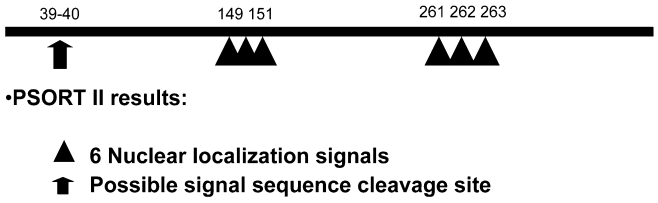


Fig. 8 Schematic representation of Rsf1p showing features predicted by PSORT II analysis. Neither a mt transit sequence nor a standard DNA-binding region were identified in this analysis

been examined. Expression of *GUT1* is positively governed via Adr1p and Ino2p/Ino4p (Grauslund et al. 1999). For *GUT2*, positive transcriptional control requires the Snf1p protein kinase and the Hap1p/Hap2p/Hap3p/Hap4p complex (Grauslund and Rønnow 2000). Both genes are negatively regulated by Opi1p (Grauslund et al. 1999; Grauslund and Rønnow 2000). UASs for the positive regulators of both *GUT1* and *GUT2* were defined. We performed an extensive computer search of the 5′-flanking regions of both *GUT1* and *GUT2* to try to identify DNA sequences that might function as a UAS for Rsf1p, but nothing emerged from these analyses other than the UASs for the known activators. Similarly, we searched the upstream flanking regions of *MRP13* and *COX4*, both of which also appear to be positively regulated by Rsf1p, for potential UASs, but again nothing compelling emerged. Thus, we are investigating possible Rsf1p–DNA interaction in these upstream flanking regions directly using standard molecular methods. However, it seems likely that Rsf1p does not interact directly with DNA sequences immediately adjacent to *GUT1*, *GUT2*, *COX4*, or *MRP13*. Rather, we suspect that it influences the production of other transcriptional activator/modulators and therefore acts at a point upstream of direct control of *GUT1* and *GUT2* expression. We have begun to address this issue using microarray analysis. Preliminary data from these experiments indicate that, in the glycerol-grown *rsf1* mutant, expression of many mt-related nuclear genes in addition to *MRP13* and *COX4* is attenuated, including that of *COX8*, *NUC1*, and others. Interestingly, these initial studies also showed a more than 5-fold down-regulation of expression from *ADR1*, the positive transcriptional regulator of *GUT1*, in the glycerol-grown *rsf1* mutant. We are repeating these array analyses and confirming the transcriptional phenotype for several nuclear genes in the *rsf1* mutant, using real-time RT-PCR. Nonetheless, these initial data strongly indicate that Rsf1p functions in the nucleus as a general transcription factor governing expression of a large panel of respiration-related genes, not only those that, like *GUT1* and *GUT2*, are involved directly in glycerol utilization.

Regardless of whether Rsf1p directly or indirectly governs expression of *GUT1*, *GUT2*, and other nuclear genes, the lack of an obvious DNA-binding region in the protein may indicate that this gene product cannot mediate gene expression by itself (but see below). Rather, it may require interaction with one or more other proteins to exert its transcriptional effects. An earlier, and quite extensive, two-hybrid study of all protein–protein interactions in *S. cerevisiae* did not identify any such interaction for Rsf1p (Uetz et al. 2000), but those analyses were performed on cells grown in glucose-based medium. Cellular cAMP levels are low in glucose-grown yeast cells (McEntee et al. 1993) and if Rsf1p, or perhaps more likely its partner(s), must be phosphorylated by PKA in order for interaction to occur, that interaction might have been missed. Moreover, *RSF1* and perhaps its interacting partners is/are expressed only at low level during glucose-based growth. We are currently performing two-hybrid analyses, using the *RSF1* coding sequence as bait and cells grown on glycerol as sole carbon source, to determine whether the product of this gene undergoes protein–protein interaction as a precondition of its role in transcriptional modulation.

RSF1 was identified in a one-hybrid screening procedure that presumably involved Rsf1p-binding to the GC-rich mt sequence element to activate *HIS3* expression. Mt transcript levels from *OLI1* and the 21S rRNA genes, both of which have copies of the mt sequence element in their 5′-flanking sequences, are attenuated significantly in the glycerol-grown *rsf1* mutant. Importantly, real-time PCR analyses showed that those low *OLI1* and 21S rRNA transcript levels originate in the glycerol-grown *rsf1* mutant from wild-type levels of mt DNA, consistent with the demonstration given here that the mutant expresses normal levels of *MIP1* mRNA. The mt *COX3* (*OXI2*) gene lacks a copy of the putative mt UAS in its upstream flanking region and one would therefore predict that, if Rsf1p is involved in mt transcriptional control via the mt sequence element, then transcript levels from this gene would remain roughly at control levels in the *rsf1* mutant during respiratory growth. Real-time RT-PCR assessing transcript levels from *COX3* supports this contention. In these same RNA/cDNA preparations, real-time analyses further indicated that transcript levels from *RPO41* are unaffected in the glycerol-grown *rsf1* mutant, and lack of the mt RNA polymerase catalytic subunit therefore cannot explain the low transcript levels from *OLI1* and the 21S rRNA genes observed in these experiments. For these and other reasons, we believe that Rsf1p is involved directly in mt transcriptional control, rather than acting at some point upstream of the immediate organellar events to influence that process. We are initiating gel-shift analyses to confirm that Rsf1p is able to undergo protein–DNA interaction with the mt UAS. Regardless, the data presented here demonstrate this protein is involved importantly in transcriptional regulation for many mt genes in yeast and for what our initial data indicate to be a large panel of mt-related nuclear genes. Thus, the *RSF1* gene product may function in the coordinate regulation of expression, not only for respiration-related nuclear genes, but also in the coordinate

transcriptional control of such genes in both nucleus and mitochondria.

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