The Aconitase Function of Iron Regulatory Protein 1

GENETIC STUDIES IN YEAST IMPLICATE ITS ROLE IN IRON-MEDIATED REDOX REGULATION*

Janaki Narahari,* Rong Ma, Man Wang, and William E. Walden§
From the Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, Illinois 60612

Iron regulatory proteins (IRP) are sequence-specific RNA-binding proteins that mediate iron-responsive gene regulation in animals. IRP1 is also the cytosolic isoform of aconitase (c-aconitase). This latter activity could complement a mitochondrial aconitase mutation (aco1) in Saccharomyces cerevisiae to restore glutamate prototrophy. In yeast, the c-aconitase activity of IRP1 was responsive to iron availability in the growth medium. Although IRP1 expression rescued aco1 yeast from glutamate auxotrophy, cells remained growth-limited by glutamate, displaying a slow-growth phenotype on glutamate-free media. Second site mutations conferring enhanced cytosolic aconitase-dependent (ECA) growth were recovered. Relative c-aconitase activity was increased in extracts of strains harboring these mutations. One of the ECA mutations was found to be in the gene encoding cytosolic NADP⁺-dependent isocitrate dehydrogenase (IDP2). This mutation, an insertion of a Ty delta element into the 5' region of IDP2, markedly elevated expression of Idp2p in glucose media. Our results demonstrate the physiological significance of the aconitase activity of IRP1 and provide insight into the role of c-aconitase with respect to iron and cytoplasmic redox regulation.

The iron regulatory proteins (IRP)† are a small family of sequence-specific RNA-binding proteins that mediate gene regulation by binding to iron-responsive elements (IRE) located in either the 5'- or 3'-untranslated region of a variety of animal cell mRNAs. IRE-containing mRNAs encode proteins of iron storage and transport as well as proteins involved in iron utilization or intermediary metabolism (for review see Refs. 1 and 2). IRPs exert their effect through translational regulation (for ferritin, m-aconitase, and erythroid aminolevulinate synthase (eALAS) mRNAs) or by controlling mRNA stability (for transferrin receptor (TfR) mRNA), depending on the location of the IRE (1–9). Two IRPs have been identified to date, called IRP1 and IRP2 (4–7). Both bind similar IRE sequences and appear to be capable of mediating iron-responsive gene regulation, although each has a distinct IRE preference (8–10). IRP1 is a bifunctional protein, having activity as an IRE-binding protein (IRE-BP) or as the cytosolic isoform of aconitase (c-aconitase) (1, 11). The two activities of IRP1 are mutually exclusive (1, 11–13). Interconversion of IRP1 between an IRE-BP and c-aconitase is itself regulated by iron, through the assembly/disassembly of a [4Fe-4S] cluster (1, 11–13). Fe-S cluster assembly occurs under conditions of excess iron, converting IRP1 to c-aconitase and stimulating synthesis of ferritin, m-aconitase, and eALAS, while TfR synthesis is depressed. Iron depletion promotes cluster disassembly, conversion of c-aconitase to IRE-BP, and repression of ferritin, m-aconitase, and eALAS synthesis, while stimulating TfR expression. In contrast to IRP1, IRP2 has only the IRE binding activity and is regulated by iron through protein degradation via the proteasome pathway (1, 14–16).

The control of IRP activity is also subject to regulation by other factors. Both IRP1 and IRP2 are phosphorylated in vivo, apparently through the action of protein kinase C (PKC) (17, 18). Treatment of cells with PKC stimulators results in an increase in IRE binding activity and TfR mRNA abundance concomitant with an increase in IRP phosphorylation (17, 18). The Fe-S cluster may be the target of phosphoregulation of IRP1. Mutation of serine 138 of IRP1, a site of PKC phosphorylation, to amino acids that mimic phosphoserine results in an increase in IRE binding activity and TfR mRNA abundance (18). Treatment of cells with PKC stimulators results in an increase in IRE binding activity and TfR mRNA abundance concomitant with an increase in IRP phosphorylation (17, 18). The Fe-S cluster may be the target of phosphoregulation of IRP1. Mutation of serine 138 of IRP1, a site of PKC phosphorylation, to amino acids that mimic phosphoserine results in oxygen-dependent Fe-S cluster instability (19). This observation suggests that the Fe-S cluster of phosphorylated c-aconitase is more susceptible to cluster disassembly in response to oxidants, which is likely to be part of the normal mechanism of cluster turnover in IRP1 (19). Exposure of cells to nitric oxide (NO) or hydrogen peroxide enhances IRE binding activity and inhibits c-aconitase activity in animal cells (reviewed in Ref. 2). Both NO and H₂O₂ cause Fe-S cluster disruption (20, 21). The consequence of these regulatory processes would be to modulate the expression of genes regulated by IRP1 and c-aconitase activity, similar to the effect of iron on IRP1.

The dichotomy between the role of IRP1 in the post-transcriptional regulation of genes involved in iron metabolism and its direct function as an enzyme of intermediary metabolism is intriguing. To gain insight into the significance of c-aconitase activity in vivo, we expressed IRP1 in aconitase-deficient (aco1) Saccharomyces cerevisiae and investigated conditions that affected its ability to provide c-aconitase activity for cell growth. We found that hyperexpression of cytosolic NADP⁺-dependent isocitrate dehydrogenase (Idp2p) enhanced the ability of IRP1 to provide aconitase function to aco1 yeast and altered the extent of interconversion of IRP1 between IRE-BP and c-acon-
Physiological Role of Cytosolic Aconitase

**Experimental Procedures**

**Strains and Growth Conditions**—The aconitase-deficient strain used throughout this study, 1150 (a, ura1-1, ura3-52, ade2-101), was constructed from a cross between MO-11-48c (a, adh1-1; (22)) and YPH500 (a, ura3-52, leu2-D1, his3-D200, trp1-D63, ade2-101, lys2-801; (23)). Yeast were transformed using the lithium acetate method (24). Transformed yeast were grown at 30 °C in complete synthetic medium supplemented with 2% glucose (25). Specific nutrients were omitted as necessary for selection and maintenance of transformants. Where indicated, glutamate was omitted to test for aconitase function in vivo. Iron-limiting media were prepared by adding the ferrous iron chelator bathophenanthroline sulfonate (BPS) (26). Nontransformed yeast strains were maintained on yeast extract/peptone/dextrose (YPD) media unless otherwise indicated (25). Yeast strain MO-11-48c was obtained from the Yeast Genetic Stock Center (Berkeley, CA), and strain YPH500 was kindly provided by Susan Liebman (University of Illinois at Chicago).

**Plasmid Constructs**—Constitutive expression of IRP1 in yeast was achieved by placing the rabbit IRSF1 cDNA (27) downstream of a minimum yeast alcohol dehydrogenase I (ADHI) promoter, obtained from plasmid pAA15 (28, 29). Plasmid pYADFRP, which carries the chimeric ADH1/IRP1 gene, was cloned into the yeast shuttle vector pRS316 (23) to yield pYRS16 (23). To express IRP1 from a low copy vector, the ADH1/IRP1 chimeric gene on pYADFRP was excised as a SpeI fragment and ligated into the SpeI site of plasmid pRS136 (23) to generate pYLC6.

**IRE** was defined as a region of the luciferase gene encoding rabbit IRSF1, with the recognition element in pYADFRP. To express IRP1 from the ADH1 promoter, the ADH1/IRP1 chimeric gene was cloned from pYADFRP, excised as a SpeI fragment, and ligated into the SpeI site of plasmid pRS136 (23) to generate pYLC6.

**Plasmid Constructs**—Constitutive expression of IRP1 in yeast was achieved by placing the rabbit IRSF1 cDNA (27) downstream of a minimum yeast alcohol dehydrogenase I (ADHI) promoter, obtained from plasmid pAA15 (28, 29). Plasmid pYADFRP, which carries the chimeric ADH1/IRP1 gene, was cloned into the yeast shuttle vector pRS316 (23) to yield pYRS16 (23). To express IRP1 from a low copy vector, the ADH1/IRP1 chimeric gene on pYADFRP was excised as a SpeI fragment and ligated into the SpeI site of plasmid pRS136 (23) to generate pYLC6.

**RII mutants** were constructed by site-specific mutagenesis, changing the codons encoding cysteines at position 437 or 503 in IRSF1 to ones encoding serine using the Altered Sites Mutagenesis kit (Promega). pGEMACO1 was generated from genomic DNA of a wild-type yeast strain using polymerase chain reaction (PCR) with the Expand Long Template PCR system (Roche Molecular Biochemicals). pGEMACO1 was generated by cloning the 3.1-kbp-amplified ACO1 gene into pGEM-T-Easy using TA cloning (Promega). Restriction enzyme analysis as well as DNA sequencing verified the authenticity of the cloned gene. A BamHI to SacI fragment from pGEMACO1 containing the complete ACO1 gene was cloned into the yeast shuttle vector pRS316 (23) to yield pRSACO1.

**To construct a luciferase reporter gene encoding mRNA containing an IRE, the following complementary oligonucleotides were annealed and ligated into the EcoRV and HindIII sites in the basic pGL3 vector (Promega): 1) 5′-GATCCTCCTGCTACACATGGTGTGACCAGAACGA-3′; 2) 5′-AGGTTCCCTGGCCCTCACTGCGGAGCAAGA-3′.**

This placed the IRE encoding sequence into the 5′-untranslated region of the luciferase gene. Luciferase gene constructs with or without the IRE were excised from the pGL3 vector as a SmaI to SalI fragment and cloned into the EcoRV to SalI sites of pRSAD1C1, placing the genes under the transcriptional direction of the minimal ADHI promoter on pRS313 (23). The plasmid encoding the luciferase gene containing the IRE is called pYahb3. The plasmid without the IRE sequence is called pYaa6.

**PCR Amplification of IRE**—Genomic DNA for use in PCR analysis of IRP1 gene structure was prepared from 1-ml overnight cultures of the strain of interest. Yeast were washed with 1 ml of sterile water and resuspended in 200 μl of water, and 400 μl of lysis buffer (10 mM Tris-HCl, pH 8.3; 50 mM KC1; 2.5 mM MgCl2; 0.1 mg/ml gelatin; 0.45% Nonidet P-40; 0.45% Tween 20; 60 μg/ml proteinase K) was added to each tube followed by incubation at 55 °C for 1 h. Proteinase K was inactivated by heating at 95 °C for 5 min, and cell debris was removed by centrifugation at 14,000 rpm for 10 min. The supernatant, which contained the genomic DNA, was used to PCR-amplify a 914-bp region of the IRP1 gene from —381 to +533 (relative to the AUG translation start codon (31)) using the following primers: primer 1, 5′-CAGGTTAGCGGGGAGGGTTTGAGACAGTTGGGACA-3′; primer 2, 5′-ACCCGACATCTTCTGGGTCTACCTGACATTCA-3′.

**Genomic Library Construction and Screening**—Genomic DNA was prepared using Promega’s genomic DNA isolation kit according to the manufacturer’s protocol. Genomic DNA was partially digested with Sau3AI and size-fractionated on a 10–40% sucrose gradient by centrifugation at 22,000 rpm for 22 h at 20 °C in a SW 50.1 rotor. After fractionation, genomic DNA (average size 12 kb) was concentrated by ethanol precipitation, ligated into the yeast shuttle vector YCP50 at the BamHI sites, and transformed into E. coli strain XLI-Blue MRF’ (Stratagene).

The genomic library was screened for genes conferring enhanced c-onitase function by transformation into the slow growing aco1 strain, 1103a (aco1, ura3-52, his3-D200, trp1-D63, ade2-101), which had been transformed with IRSF1 placmid. A total of 120,000 transformants were screened. Because of the relatively high background of spontaneous fast-growers generated relative to the frequency with which the mutant gene was expected to occur in transformants, we developed a routine method to eliminate fast-growing transformants that had resulted from spontaneous chromosomal mutations. Transformants were first plated onto media lacking glutamate and histidine, and fast-growing strains were selected. These were then replica-plated onto selective media lacking glutamate and uracil. Here only cells that had taken up a library clone would grow, and only those that had acquired a gene conferring fast growth would grow rapidly. Transformants that fell into this category were then counterselected on media lacking glutamate and containing 5-fluoro-orotic acid (FOA) (32). Cells that required the genomic library clone for fast growth would fail to grow rapidly on FOA media and were likely candidates for having acquired a gene from the library that conferred fast growth. Of the 120,000 transformants, 160 grew rapidly on media lacking glutamate and histidine, and only 4 of these transformants also required the library clone for fast growth. These four transformants were analyzed further.

**Preparation of Yeast Cytoplasmic Extracts and Enzyme Assays**—The preparation of yeast cytoplasmic extracts and performance of aconitase assays was as described elsewhere (19). Extracts for NADP+-dependent isocitrate dehydrogenase assay were made, and assays were performed as described in Ref. 31.

**Protein Immunoblot**—Aliquots of cytoplasmic extracts (15 μg) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes using a Trans-blot semi-dry transfer unit (Bio-Rad). The membrane was blocked with 5% nonfat milk in phosphate-buffered saline. These results provide insight into the possible role of c-onitase in animal cells.

![Fig. 1. Analysis of growth of IRSF1-transformed aco1 yeast. aco1 yeast was transformed with a chimeric gene encoding rabbit IRSF1 (1150-IRP1) or with the yeast ACO1 gene (1150-ACO1). Cells from a fresh overnight culture, grown in selective medium supplemented with glutamate, were washed twice with sterile water and resuspended into 1 ml sorbitol. Cells were serially diluted into 1 ml sorbitol to give from 5 × 10^2 to 50 cells in 10 μl, which were spotted onto selective medium with or without glutamate as indicated, and without (–) or with (+) BPS (indicated on the right). Where added, BPS was included at 50 μM to reduce available iron in the medium. Shown is growth after 5 days of incubation at 30 °C.](image)
The addition of iron to yeast culture media did not result in an increase in c-aconitase activity. However, depletion of available iron in the medium by addition of the ferrous iron chelator BPS did cause reduction in aconitase activity (Table I). Subsequent addition of excess iron to iron-depleted medium restored c-aconitase activity. Given the inhibition of c-aconitase by iron depletion, the effect of limiting iron on cell growth was examined. Inclusion of 50 μM BPS in growth media supplemented with glutamate did not significantly effect growth of 1150-IRP1 (Fig. 1). In sharp contrast, growth of these yeast on glutamate-free medium was strongly inhibited by 50 μM BPS. The addition of this amount of BPS did not inhibit growth of cells expressing m-aconitase (ACO1) with or without glutamate supplementation (Fig. 1). The correspondence between the effects of BPS on growth of 1150-IRP1 and c-aconitase activity (Table I) suggests that cluster assembly or disassembly in IRP1 is responsive to cellular iron status and that this sensitivity is greater than that of m-aconitase or other iron-requiring activities in yeast.

Isolation of Mutants of 1150-IRP1 Displaying Fast Growth on Glutamate-free Media—To investigate the nature of the growth limitation of 1150-IRP1 in the absence of glutamate, we screened for spontaneous fast-growing mutants on glutamate-free medium. Colonies of 1150-IRP1 normally require at least 7–10 days to appear as very small colonies on media lacking glutamate. A total of 25 colonies appeared in 4–5 days on lawns of 1150-IRP1 replica-plated onto media lacking glutamate. The

**RESULTS**

**Complementation of Yeast aco1 by IRP1—Aconitase deficiency leads to glutamate auxotrophy in yeast** (22). The aco1 yeast strain 1150 was transformed with a plasmid designed for constitutive expression of IRP1 and tested for growth on glutamate-free media. Results from a representative transformant clone (called 1150-IRP1) are presented. Expression of IRP1 in this strain restored glutamate prototrophy, although growth in the absence of glutamate was not as vigorous as in glutamate-supplemented media (Fig. 1). In contrast, restoration of wild-type mitochondrial aconitase by transformation with the yeast ACO1 gene resulted in vigorous growth in the presence and absence of glutamate (Fig. 1). To investigate whether it was the c-aconitase activity of IRP1 that was responsible for the rescue from glutamate auxotrophy, we constructed IRP1 mutants C437S and C503S and used them to transform the aco1 yeast. Cys-437 and Cys-503 have been implicated as ligands for the Fe-S cluster in IRP1 and therefore are predicted to be indispensable for aconitase function (34, 35). Strain 1150, expressing either of these mutant IRP1s, failed to grow on glutamate-free media (not shown).

The aconitase activity in extracts of IRP1-transformed aco1 yeast was also examined. Aconitase activity was elevated greater than 20-fold in extracts of aco1 yeast expressing IRP1 than in nontransformed cells or yeast transformed with the C437S IRP1 mutant (Table I). To determine the amount of IRP1 that could function as c-aconitase, extracts were subjected to conditions promoting Fe-S cluster reconstitution and assayed for aconitase activity (36). Activity in extracts from 1150-IRP1 was elevated approximately 8-fold by this treatment, whereas the activity in nontransformed 1150 was unaltered (not shown). These results indicate that about 12% of the total amount of IRP1 is present as active c-aconitase in 1150-IRP1.

**TABLE I**

| IRP1 | Media additions | Aconitase activity (mU/mg extract) |
|------|----------------|----------------------------------|
|      | normal         | BPSa                            | BPS + ironb                 |
| wt   | 13.1           | 2.6                             | 5.9                          |
| None | 0.5            | ndc                            | nd                          |
| C437S| 0.4            | ndc                            | nd                          |

a 100 μM BPS.
b 100 μM BPS followed by 1 mM FeSO4.
c Not determined.

dec (!((100μM) \(FeSO_4\), normal (0.5 mg protein) or in this medium supplemented with 100 μM BPS or 100 μM BPS followed by 1 mM FeSO4, as indicated. When added, BPS was incubated with cells for 16 h. Iron was added during the last 4 h of incubation with BPS. Aconitase assays were performed as described under "Experimental Procedures." Data are an average of two independent experiments. Strain 1150 was transformed with IRP1 on pYADFRP (wt), with vector only (None), or with a plasmid expressing the C437S IRP1 mutant (C437S).

**FIG. 2.** Growth characteristics of IRP1-transformed mutant aco1 yeast on media lacking glutamate. Strain 1150 transformed with pYADFRP was grown overnight in YPD medium, and aliquots containing approximately 10^6 cells were spread onto the YPD plates. After a further overnight incubation, cells were replica-plated onto selective media lacking glutamate. Twenty-five colonies appeared as colony outgrowths in a screen of 10 plates. Shown is the growth after 5 days, + and − glutamate (as indicated), of six of the resulting strains isolated from this screen. These strains represent the range of growth characteristics seen among the isolated strains.
FIG. 3. Determination of c-aconitase activity in IRP1-transformed mutant aco1 yeast. Yeast cells were grown to mid-logarithmic phase in selective media supplemented with glutamate. At this point, cells were harvested and cytoplasmic extracts were prepared and analyzed for aconitase activity. Activity is expressed in milliunits per mg of total extract protein. Error bars indicate the standard deviation of results from three experiments.

FIG. 4. Sensitivity of IRP1-dependent growth to iron depletion. Strains 1150 (●), ECA121 (○), ECA122 (×), and ECA125 (■), each expressing IRP1, were grown in synthetic media supplemented with glutamate and 100 μM BPS for 18 h, washed twice with de-ionized water, and transferred to media containing varying concentrations of BPS, with and without glutamate. Cell growth was monitored at A600.

Analysis of IRP1-dependent Aconitase Activity in ECA Strains—The aconitase activity in extracts of the most robust ECA strains (ECA121, ECA122, and ECA125) was examined. Aconitase activity measured from each strain was markedly higher than that measured in extracts of the parent, 1150-IRP1 (Fig. 3). We estimate that 20–40% of IRP1 was active as c-aconitase in extracts from these ECA strains compared with about 12% in extracts of the 1150-IRP1 strain. Glutamate pools were also significantly increased in these ECA strains in comparison to 1150-IRP1, consistent with their improved growth in glutamate-free media and increased aconitase activity (not shown). These results suggest that IRP1 was either converted to a higher rate to c-aconitase or that c-aconitase was more stable in these ECA strains.

Differential Sensitivity of ECA Strains to Iron Depletion—The ECA strains were examined for the sensitivity of aconitase-dependent growth to iron depletion caused by addition of BPS to the growth medium. Cytoplasmic aconitase activity and c-aconitase-dependent growth of 1150-IRP1 was sensitive to iron deprivation (Fig. 1 and Table I). To examine the effect of iron deprivation on c-aconitase-dependent growth quantitatively, growth in the presence of BPS was measured in liquid culture. Specific effects of iron deprivation on IRP1 function were revealed by examining the ratio of exponential growth rates in the absence and presence of glutamate (relative growth rate) as a function of BPS concentration. The relative growth rate for 1150-IRP1 showed a gradual but steady decline with increasing BPS concentration from 0 to 50 μM (Fig. 4). No inhibition of growth rate was observed in yeast grown in glutamate-supplemented media with ≤20 μM BPS (not shown). Maximum inhibition of relative growth rate was attained at 50 μM BPS. Addition of ≥50 μM BPS to the growth medium depressed growth rate in the presence and absence of glutamate, although inhibition was stronger in the absence of glutamate.

The effect of BPS on growth of the ECA strains showed striking differences. The relative growth rate of ECA122 and ECA125 was maximally inhibited at 20 μM BPS (Fig. 4). In media supplemented with glutamate, no inhibition of growth of ECA122 or ECA125 was detected at 20 μM BPS, whereas growth in the absence of glutamate was inhibited by close to 70%. These results suggest that the ECA phenotype of these strains, and perhaps the enhanced c-aconitase activity itself, also exhibit enhanced iron dependence relative to other cellular activities. In contrast, growth of ECA121 was slightly more resistant to iron depletion than the 1150-IRP1 strain and significantly more resistant than either ECA122 or ECA125.
Identification of a Yeast IDP2 Gene Mutation That Confers the Fast Growth Phenotype—The ECA125 strain was selected to begin the identification of genes that conferred the fast growth phenotype. We first examined the inheritance of the fast growth phenotype of this strain. ECA125 was cured of the plasmid carrying the IRP1 gene and then crossed with YPH500, an ACO1 strain. The resulting diploid was sporulated, tetrads were dissected, and haploid yeast strains were generated upon germination of the resulting spores. Segregation of aco1 was 2:2 in the haploid progeny. Sixty-eight aco1 strains resulting from this process were transformed with IRP1 and examined for growth on glutamate-free medium. Forty-two of the aco1 strains gave rise to transformants displaying the fast-growth phenotype similar to ECA125, whereas the remaining 26 strains yielded transformants with the slow-growth phenotype similar to the 1150-IRP1 strain. The higher number of fast-growing aco1 strains suggests a linkage between aco1 and the gene mutated to give the fast-growth phenotype. Matings between aco1 haploid strains revealed that the mutation conferring the fast-growth phenotype was dominant.

To identify the mutant gene in this strain, a genomic library was constructed using DNA isolated from one of the spore strains, and the library was used to transform a slow growth, IRP1-transformed aco1 strain to fast-growth (see “Experimental Procedures”). Four fast-growth transformants that were dependent on IRP1 and the resident genomic clone were obtained in a screen of 120,000 transformants. Restriction endonuclease patterns obtained for each of these clones suggested that they were overlapping (not shown).

The ends of the insert in the genomic clone containing the smallest insert were sequenced and compared with the yeast genome. One end of the genomic fragment showed identity to the 3′-end of the gene encoding an isoform of NADP⁺-dependent, isocitrate dehydrogenase (IDP2), the cytosolic isoform of isocitrate dehydrogenase (31). Surprisingly, the sequence at the other end of the 2-kbp genomic clone showed homology with Ty delta elements (37), and not to the 5′-end of the IDP2 gene. The fact that neither Ty nor delta elements have been found in the yeast genome at the position predicted by this clone suggests that an insertion of a Ty element in the 5′ region of the IDP2 gene occurred in the ECA125 strain.

PCR primers were generated to probe the 5′ region of the IDP2 gene in ECA125 and other ECA strains. Amplification of parental 1150 DNA or DNA of a wild type yeast strain (YPH500) yielded the predicted 914-bp-long fragment, nucleotides −381 to +533 relative to the IDP2 translation start codon (Fig. 5). The PCR product from ECA125 was larger, approximately 1.3 kbp long, indicating the presence of an approximately 400-bp insert (Fig. 5). Amplification of DNA from ECA122 indicated a similar insert, whereas strain ECA121 gave the 914-bp fragment seen with DNA from 1150 and wild-type yeast (Fig. 5). Examination of the other ECA strains for the presence of this insert in the IDP2 gene showed that most of the other ECA strains gave the 914-bp product seen with strain 1150 DNA. However, seven of these ECA strains gave the 1.3-kbp-long amplified product, indicating the presence of the −400-bp insert in the 5′ region of the IDP2 gene (not shown) and suggesting that these isolates may not be independent.

Analysis of Expression of Idp2p in ECA Strains Carrying the IDP2 Insertion Mutation—It has been documented that insertion of the yeast retrotransposon Ty into promoter regions can alter gene expression (37). To investigate the effect of the Ty insertion mutation on Idp2p expression, the relative level of Idp2p was determined by protein immunoblot (see “Experimental Procedures”). A clear band corresponding to Idp2p was seen in extracts of strains harboring the mutant IDP2 gene, such as ECA122 and ECA125 (Fig. 6A). This protein band was undetectable in extracts of ECA121 or the parental 1150 strain (Fig. 6A). Idp2p was undetectable in other ECA strains that lacked the 400-bp insert, whereas all strains that carried the insertion mutation in IDP2 showed variable but highly elevated expression of Idp2p (not shown).

We measured NADP⁺-dependent isocitrate dehydrogenase activity in unfractionated yeast extracts. A basal level of activity in all strains reflects the constitutive expression of Idp1p, the mitochondrial isoform of NADP⁺-dependent isocitrate dehydrogenase (38) (Fig. 6A). ECA strains harboring a normal IDP2 gene (such as ECA121) gave enzymatic activity levels that were similar to 1150-IRP1 (Fig. 6B). In contrast, NADP⁺-dependent isocitrate dehydrogenase activity was elevated significantly in strains in which Idp2p was up-regulated. For example, this activity was nearly 4.5-fold higher in extracts of ECA125 and more than 2-fold higher in ECA122 extracts when compared with this activity in extracts of the parental 1150-IRP1 strain (Fig. 6B). These results confirm that the insertion mutation in the IDP2 gene causes an increase in Idp2p expression and activity and suggest that elevated Idp2p activity pro-
motes enhanced IRP1-dependent c-aconitase activity.

The IDP2 Mutation Segregates with the Fast-growth Phenotype in aco1 Yeast—
Haploid strains obtained from the cross of ECA125 with YPH500 were analyzed for the segregation of the IDP2 mutation and elevated expression. Fig. 7A shows a protein immunoblot of Idp2p in extracts from the four spores of one tetrad. Idp2p, which is expressed constitutively, is also shown. B, the total NADP⁺-dependent isocitrate dehydrogenase activity was measured by following the production of NADPH at 340 nm as described under “Experimental Procedures.” This assay measures activity from Idp1p as well as Idp2p. Error bars, standard deviation.

The dual activities of IRP1 are mutually exclusive and so an enhancement of c-aconitase is expected to coincide with a decrease in IRE binding activity (1, 2). To investigate how the hyperexpression of Idp2p affected IRP1 function as an IRE-binding protein in vivo, we examined IRP1-mediated translational repression in strains generated from spores b and d shown in Fig. 7A. The two aco1 spores of the tetrad were analyzed for IRP1-dependent growth. Spore d, which harbors the mutation in IDP2, grew much faster than spore b, which lacks the mutation (data not shown).

The role of IRP1 as c-aconitase in animal cells has been overshadowed by the focus on its function as a regulator of gene expression. In fact, IRP1 exists predominantly as c-aconitase in some tissues, particularly liver (11, 39). Reconstituted in yeast, the ability of IRP1 to function physiologically to provide c-aconitase activity is evident. Here we show that a mutation that leads to hyperexpression of cytosolic isocitrate dehydrogenase enhances the ability of yeast to utilize IRP1 as c-aconitase. Because animal cells also have a cytosolic isocitrate de-
hydrogenase, c-aconitase in animal cells may well contribute normally to glutamate biosynthesis and other metabolic processes such as fatty acid metabolism (40–43).

Depletion of available iron in yeast growth media inhibited c-aconitase activity and growth of IRP1-transformed aco1 yeast on glutamate-free media. That iron may regulate pathways involving c-aconitase in animal cells raises questions regarding the role and significance of iron in regulating metabolic pathways involving c-aconitase. In addition to converting isocitrate to α-ketoglutarate, which is a precursor in glutamate biosynthesis (38), the reaction catalyzed by Idp2p also produces NADPH. Idp2p is an important source of this cofactor in the cytosol (40, 42–44). NADPH is a key cofactor in cellular defenses against oxidative stress, particularly through its involvement in the thioredoxin and glutathione redox cycles (44–46). We propose a model whereby regulation of IRP1/c-aconitase by iron coordinates NADPH levels with iron uptake, utilization, and storage. This would provide the cell with the reducing power to deal with the increased oxidative stress brought on by higher intracellular iron and an additional source of NADPH as a cofactor for ferric reductase (47, 48). Ferric reductase has been shown to be an important component of iron transport systems in eukaryotes (49–54). The increase in NADPH predicted to accompany the rise in c-aconitase activity in iron replete cells would provide additional reducing equivalents, allowing animal cells to maintain the redox balance in the cytoplasm during intensive iron transport. Moreover, the effect of iron on c-aconitase activity and downstream steps catalyzed by Idp2p would provide animal cells with a means to modulate NADPH production specifically. Increased c-aconitase-driven NADPH levels also would favor the Fe(II) state and thereby promote ferritin-mediated iron biomineralization and iron incorporation into heme (55, 56). The evolution of c-aconitase as an iron-responsive regulator of ferritin synthesis may have been prompted by this redox-dependent regulation of iron storage and utilization.

It is not surprising that a mutation in the gene encoding the cytosolic isoform of isocitrate dehydrogenase alters glutamate synthesis in cells utilizing IRP1 as c-aconitase. Idp2p most likely drives the reactions toward glutamate by mass action in Idp2p-hyperexpressing cells. On the other hand, we would have predicted that hyperexpression of Idp2p would not effect the interconversion of IRP1 between the IRE-BP and c-aconitase. However, a higher percentage of IRP1 was converted to c-aconitase in strains that hyperexpressed Idp2p. This suggests that the increase in Idp2p activity either enhanced conversion of IRP1 to c-aconitase or stabilized c-aconitase once it was formed. At present, we cannot distinguish between these possibilities. The proportion of IRP1 that exists as c-aconitase is affected by oxidants produced during normal, aerobic metabolism in yeast (19). Therefore, it is possible that the anti-oxidant effects of elevated NADPH may have protected c-aconitase in these yeast. Alternatively, conversion of apo-IRP1 to c-aconitase could have been enhanced in these strains. Elevated NADPH would effect levels of reduced thioredoxin, which has been shown to reduce oxidized apo-IRP1 generated upon Fe-S cluster removal (57). Protein thiol reduction appears to be a necessary step in the assembly of Fe-S clusters in aconitases (36). Increased NADPH may also enhance Fe-S cluster assembly by increasing availability of Fe(II).

In animal cells, interconversion of IRP1 and c-aconitase and iron-responsive gene regulation respond to chelatable iron levels (58). In yeast, the transcription factor Aft1p responds to chelatable iron levels by tightly regulating iron uptake by controlling the expression of genes encoding the components of the high affinity iron transport system (59–61). Therefore, we might expect that overexpression of IRP1, which could deplete the chelatable iron pool, would cause a net increase in iron accumulation in yeast. We did not observe a consistent increase in iron accumulation in strains overexpressing IRP1, suggesting that iron was not the limiting factor for cluster assembly in IRP1 in yeast (not shown). On the other hand, assembly of an Fe-S cluster in IRP1 in the cytosol of yeast could be limiting. We cannot rule out this possibility at the present time, but other cytosolic Fe-S proteins do exist in yeast, and so it is expected that the machinery for assembling Fe-S clusters in cytosolic proteins is present (62).

Growth of aco1 strains that hyperexpress Idp2p on glutamate-free media was very sensitive to the level of c-aconitase activity. This was most evident when iron availability was reduced in the growth media, a condition that significantly inhibited c-aconitase activity (Table I). These yeast strains were hypersensitive to iron depletion, in fact, suggesting that growth of these strains became limited by c-aconitase activity in low iron media. We have also observed effects on growth of an Idp2p hyperexpressing strain when expressing IRP1 mutants that have defects in c-aconitase function (19). Mutations in IRP1 that decreased c-aconitase activity in vivo strongly reduced growth of these yeast strains on glutamate-free media. Moreover, reduction in c-aconitase activity in vivo by lowering IRP1 expression in these strains also led to a much slower growth rate.2 The responsiveness of the strains that hyperexpress Idp2p to the level of c-aconitase activity makes them very useful for the study of factors and conditions that affect IRP1 function in vivo.

Acknowledgments—We thank Susan Liebman for providing yeast strains and plasmids and for helpful comments during the course of this study. We also thank Dennis Thiele for providing PRS313, PRS316, and pAAH5; Lee McAlister-Henn for providing Idp1/2-specific antiserum; and David Ucker, Simon Silver, and Nina Brown for comments on the manuscript.

REFERENCES

1. Eisenstein, R. S., Kennedy, M. C., and Beinert, H. (1997) in Metal Ions in Gene Regulation (Silver, S., and Walden, W., eds) pp. 157–216, International Thomson Publishing, New York
2. Hentze, M. W., and Kühl, L. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8175–8182
3. Theil, E. (1998) in Metal Ions in Biological Systems (Sigel, A., and Sigel, H., eds) pp. 403–434, Marcel Dekker, Inc., New York
4. Samaniego, F., Chin, J., Iwai, K., Rouault, T. A., and Klausner, R. D. (1994) J. Biol. Chem. 269, 30904–30910
5. Guo, B., Yu, Y., and Leibold, E. A. (1994) J. Biol. Chem. 269, 24252–24260
6. Guo, B., Brown, F. M., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) J. Biol. Chem. 270, 16529–16535
7. Henderson, B. R., Seiser, C., and Kühl, L. C. (1993) J. Biol. Chem. 268, 27297–27304
8. Henderson, B. R., Menotti, E., and Kühl, L. C. (1996) J. Biol. Chem. 271, 4900–4908
9. Butt, J., Kim, H.-Y., Basilion, J. P., Cohen, S., Iwai, K., Philipp, C. C., Altschul, S., Klausner, R. D., and Rouault, T. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4345–4349
10. Keny, X., Wu, J., Leibold, E. A., Walden, W. E., and Theil, E. C. (1998) J. Biol. Chem. 273, 23637–23640
11. Kennedy, M. C., Mende-Mueller, L., Blondin, G. A., and Beinert, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11730–11734
12. Kapustin, S., Downey, W. E., Tang, C., Philipp, C., Haile, D., Orloff, D. G., Harford, J. B., Rouault, T. A., and Klausner, R. D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10109–10113
13. Harford, J. B., Rouault, T. A., Tang, C. C., Chin, J., Harford, J. B., and Klausner, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7536–7540
14. Iwai, K., Drake, S. K., Wehr, N. B., Weissman, A. M., LaVauite, T., Saito, N., Klausner, R. D., Levine, R., K., Rouault, T. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4924–4928
15. Iwai, K., Klausner, R. D., and Rouault, T. A. (1995) EMBO J. 14, 5350–5357
16. Brown, B., Phillips, J. D., Yu, Y., and Leibold, E. A. (1995) J. Biol. Chem. 270, 21645–21651
17. Eisenstein, R. S., Tuazon, P. T., Schalinske, K. L., Anderson, S. A., and Traugh, J. A. (1993) J. Biol. Chem. 268, 27363–27370
18. Schaefer, K. L., and Eisenstein, R. S. (1990) J. Biol. Chem. 271, 7168–7176
19. Brown, N. M., Anderson, S. A., Steffen, D. W., Carpenter, T. B., Kennedy, M. C., Walden, W. E., and Eisenstein, R. S. (1998) Proc. Natl. Acad. Sci.

2 J. Narahari, unpublished observations.
20. Kennedy, M. C., Antholine, W. E., and Beinert, H. (1997) J. Biol. Chem. 272, 20340–20347
21. Brazzolotto, X., Gaillard, J., Pantopoulos, K., Hentze, M. W., and Mouli, J.-M. (1999) J. Biol. Chem. 274, 21625–21630
22. Ogur, M., Coker, L., and Ogur, S. (1964) Biochem. Biophys. Res. Commun. 14, 163–168
23. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
24. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
25. Rose, M. D., Winston, F., and Hieter, P. (1990) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26. Askwith, C., Eide, D., van Ho, A., Bernard, P. S., Li, L., Davis-Kaplan, S., Sipe, D. M., and Kaplan, J. (1994) Cell 76, 403–410
27. Patino, M. M., and Walden, W. E. (1992) J. Biol. Chem. 267, 19011–19016
28. Bennetzen, J. L., and Hall, B. D. (1982) J. Biol. Chem. 257, 3018–3025
29. Ammerer, G. (1983) Methods Enzymol. 101, 192–201
30. Gangloff, S. P., Marguet, D., and Lauquin, G. J.-M. (1990) Mol. Cell. Biol. 10, 3551–3561
31. Loftus, T. M., Hall, L. V., Anderson, S. L., and McAlister-Henn, L. (1994) Biochemistry 33, 9661–9667
32. Boeke, J. D., Lacroute, F., and Fink, G. R. (1984) in The Molecular and Cellular Biology of the Yeast Saccharomyces (Broach, J. R., Pringle, J. R., and Jones, E. W., eds) pp. 193–261, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
33. Haselbeck, R. J., and McAlister-Henn, L. (1993) J. Biol. Chem. 268, 12116–12122
34. Philpott, C. C., Haile, D., Rouault, T., and Klausner, R. D. (1993) J. Biol. Chem. 268, 17655–17668
35. Hirling, H., Henderson, B. R., and Kuhn, L. C. (1994) EMBO J. 13, 453–461
36. Kennedy, M. C., Emptage, M. H., Dreyer, J.-L., and Beinert, H. (1983) J. Biol. Chem. 258, 11098–11105
37. Boeke, J. D., and Sandmeyer, S. B. (1991) in The Molecular and Cellular Biology of the Yeast Saccharomyces (Broach, J. R., Pringle, J. R., and Jones, E. W., eds) pp. 193–261, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
38. Zhao, W.-N., and McAlister-Henn, L. (1996) Biochemistry 35, 7873–7878
39. Chen, O. S., Schalinske, K. L., and Eisenstein, R. S. (1997) J. Nutr. 127, 1231–1239
40. Minard, K. I., Jennings, G. T., Loftus, T. M., Xuan, D., and McAlister-Henn, L. (1998) J. Biol. Chem. 273, 31486–31493
41. Jennings, G. T., Sadieir, J. W., and Stevenson, P. M. (1996) Biochim. Biophys. Acta 1304, 219–227
42. Farrell, H. M., Wickham, E. D., and Reeves, H. C. (1995) Arch. Biochem. Biophys. 321, 199–208
43. van Roermund, C. W. T., Hettema, E., H., Kal, A. J., van den Berg, M., Tabak, H. F., and Wanders, R. J. A. (1998) EMBO J. 17, 677–687
44. Minard, K. I., and McAlister-Henn, L. (1999) J. Biol. Chem. 274, 3402–3406
45. Bodaness, R. S. (1982) Biochem. Biophys. Res. Commun. 108, 1709–1715
46. Winkler, B. S., DeSantis, N., and Solomon, F. (1986) Exp. Eye Res. 43, 829–847
47. Shatwell, K. P., Dancis, A., Cross, A. R., Klausner, R. D., and Segal, A. W. (1990) J. Biol. Chem. 271, 12940–12944
48. Finegold, A. A., Shatwell, K. P., Segal, A. W., Klausner, R. D., and Dancis, A. (1990) J. Biol. Chem. 271, 31021–31024
49. Dancis, A., Klausner, R. D., Hinnebusch, A. G., and Barrio, J. (1996) Mol. Cell. Biol. 10, 2294–2301
50. Jordan, I., and Kaplan, J. (1994) Biochem. J. 302, 875–879
51. Akompong, T., Inman, R. S., and Wessling-Resnick, M. (1995) J. Biol. Chem. 270, 20997–20941
52. Inman, R. S., Coughlan, M. M., and Wessling-Resnick, M. (1994) Biochemistry 33, 11850–11857
53. Parkes, J. G., Olivier, N. F., and Templeton, D. M. (1997) Toxicology 117, 141–151
54. Han, O., Failla, M. L., Hill, A. D., Morris, E. R., and Smith, J. C. (1995) J. Nutr. 125, 1291–1299
55. Waldo, G. S., and Theil, E. C. (1996) in Comprehensive Supramolecular Chemistry (Suslick, K. S., ed) pp. 65–89, Pergamon Press, Oxford
56. Ferreira, G. C. (1999) Int. J. Biochem. Cell Biol. 31, 995–1000
57. Oliveira, L., Bouton, C., and Drapier, J.-C. (1999) J. Biol. Chem. 274, 516–521
58. Rogers, J., and Munro, H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2277–2281
59. Hassett, R. F., Romeo, A. M., and Kosman, D. J. (1998) J. Biol. Chem. 273, 7626–7636
60. Yamaguchi-Iwai, Y., Steurman, R., Dancis, A., and Klausner, R. D. (1996) EMBO J. 15, 3377–3384
61. Yamaguchi-Iwai, Y., Dancis, A., and Klausner, R. D. (1995) EMBO J. 14, 1231–1239
62. Kispal, G., Csere, P., Prohl, C., and Lill, R. (1999) EMBO J. 18, 3981–3989