EUKARYOTIC CELL, Mar. 2010, p. 460–471 Vol. 9, No. 3
1535-9778/10/$12.00 doi:10.1128/EC.00213-09
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Iron Regulation through the Back Door: Iron-Dependent Metabolite Levels Contribute to Transcriptional Adaptation to Iron Deprivation in *Saccharomyces cerevisiae*▽†

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Received 22 July 2009/Accepted 3 December 2009

Budding yeast (*Saccharomyces cerevisiae*) responds to iron deprivation both by Aft1-Aft2-dependent transcriptional activation of genes involved in cellular iron uptake and by Cth1-Cth2-specific degradation of certain mRNAs coding for iron-dependent biosynthetic components. Here, we provide evidence for a novel principle of iron-responsive gene expression. This regulatory mechanism is based on the modulation of transcription through the iron-dependent variation of levels of regulatory metabolites. As an example, the *LEU1* gene of branched-chain amino acid biosynthesis is downregulated under iron-limiting conditions through depletion of the metabolic intermediate α-isopropylmalate, which functions as a key transcriptional coactivator of the Leu3 transcription factor. Synthesis of α-isopropylmalate involves the iron-sulfur protein Ilv3, which is inactivated under iron deficiency. As another example, decreased mRNA levels of the cytochrome *c*-encoding *CYC1* gene under iron-limiting conditions involve heme-dependent transcriptional regulation via the Hap1 transcription factor. Synthesis of the iron-containing heme is directly correlated with iron availability. Thus, the iron-responsive expression of genes that are downregulated under iron-limiting conditions is conferred by two independent regulatory mechanisms: transcriptional regulation through iron-responsive metabolites and post-transcriptional mRNA degradation. Only the combination of the two processes provides a quantitative description of the response to iron deprivation in yeast.

Iron is a key trace element for virtually all organisms. It functions as an essential cofactor in central cellular processes such as respiration, DNA synthesis and repair, ribosome biogenesis, metabolite biosynthesis, and oxygen transport in vertebrates. Although iron is highly abundant, its bioavailability is low due to its poor solubility under ambient conditions. On the other hand, iron is a major source of reactive oxygen species and thus toxic at higher concentrations. Therefore, cells have developed sophisticated systems for ensuring a tightly regulated cellular iron homeostasis (23, 24, 35, 51, 53). In the budding yeast *Saccharomyces cerevisiae*, adaptation to iron-deprived conditions elicits a strong response at the level of mRNA. This response includes the downregulation of genes for components of mitochondrial respiration and the citric acid cycle; the remodeling of genes of biosynthetic pathways that include iron-dependent enzymes such as the synthesis of ergosterol, unsaturated fatty acids, biotin, purines, and several amino acids; and of genes encoding heme proteins or iron-sulfur (Fe/S) proteins (23, 35, 37, 38, 47). At the same time, genes involved in hexose acquisition, fermentation, and cellular iron acquisition are highly induced. Disruption of the mitochondrial iron-sulfur cluster (ISC) assembly and export systems elicits a similar response, indicating that both mitochondrial Fe/S protein synthesis activity and extracellular iron levels are key regulatory factors for the maintenance of cellular iron homeostasis in *S. cerevisiae* (2, 20).

Many of the iron-responsive genes that are induced upon iron deprivation are under the control of the iron-responsive transcription factors Aft1 and, to a minor extent, Aft2 (23, 35, 41, 42). The Aft1-Aft2-dependent yeast iron regulon comprises a set of ~40 genes encoding products that function mostly in iron uptake at the cell surface and in intracellular iron transport (35, 41, 42, 47). Aft1 shuttles between the cytosol and nucleus in an iron-responsive manner and acts as a transcriptional activator (52, 54). The monothiol glutaredoxins Grx3 and Grx4, the regulatory proteins Fra1 and Fra2, and a signaling molecule produced and exported by the mitochondrial ISC systems are required for proper sensing of iron by Aft1 (4, 20, 26, 33, 43).

The iron regulon includes two members of a conserved family of tandem zinc finger-containing mRNA-binding proteins, Cth1 and Cth2, which promote mRNA decay (37, 38, 53). Under iron-limiting conditions, Cth1 and Cth2 bind to specific AU-rich elements (AREs) within the 3′ untranslated region (UTR) of many mRNAs encoding proteins involved in iron-dependent pathways. The Cth proteins recruit the Dhh1 RNA helicase, which interacts with multiple members of the general machinery of mRNA decay, thus promoting the 5′-to-3′ degradation of mRNAs at cytoplasmic P bodies (34). Virtually all genes that are repressed upon iron deprivation display an aberrant expression in cells lacking *CTH1* and *CTH2*, demon-

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† Supplemental material for this article may be found at http://ec.asm.org/.

▽ Published ahead of print on 11 December 2009.

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strating the global contribution of this posttranscriptional process to the regulation of iron homeostasis in *S. cerevisiae* (53). However, the extent of downregulation of most genes upon iron deprivation is not quantitatively explained by the roles of the Cth proteins. Hence, there seems to be another important level of regulation that so far cannot be explained by Aft1-Aft2-dependent transcriptional activation and/or Cth protein-dependent mRNA degradation.

In this work we have identified the molecular basis of the Cth-independent downregulation of iron-responsive genes during the adaptation of yeast to iron deprivation. By focusing our analyses on the *LEU1* gene of the branched-chain amino acid biosynthesis pathway and on *CYC1*, encoding cytochrome *c*, we show that the downregulation of iron-dependent metabolic pathways is mediated by decreased levels of key regulatory metabolites, in these cases, α-isopropylmalate (α-IPM) and heme, respectively, under iron-limiting conditions. Decreased synthesis of α-isopropylmalate, a transcriptional coactivator of the transcription factor Leu3 regulating *LEU1* expression (13, 25, 49), is caused by the loss of function of the Fe/S protein Ilv3 under iron limitation. Likewise, the levels of the iron-containing heme are adjusted in an iron-dependent fashion. Thus, downregulation of iron-responsive genes in response to iron deprivation involves two levels of regulation in *S. cerevisiae*: transcriptional adaptation through metabolites that are synthesized in an iron-dependent fashion and posttranscriptional mRNA degradation.

MATERIALS AND METHODS

Yeast strains, cell growth, and plasmids. Yeast strains used in this study are listed in Table S1 in the supplemental material. Cells were cultivated in synthetic complete minimal medium containing all recommended supplements (SC) and 2% (wt/vol) glucose (SD) or 2% (wt/vol) raffinose (48). Repression of conditional Gal strains was performed as described in the corresponding literature (see Table S1). Plasmid-based reporter constructs for monitoring *LEU1* expression were constructed by replacing the *MET25* promoter and the *CYC1* terminator of vector p416-MET25-luc2 harboring the firefly *luc2* gene (32). Those for monitoring the expression of *CYC1* were based on vector p416-MET25-hRluc harboring *Renilla* luciferase, hRluc, or p414-MET25-luc2 (32). For further details on plasmids used in this study, see Table S2 in the supplemental material. Constructs were verified by DNA sequencing and/or functional complementation of corresponding yeast mutants.

Reporter assays. For analysis of iron-responsive gene expression using luciferase-based reporter constructs, cells were cultivated at 30°C overnight in minimal medium containing either 50 μM bathophenanthroline or 50 μM $FeCl_3$. Cells were diluted in 5 ml of the same medium to an optical density at 600 nm (OD_{600}) of 0.2, incubated until the optical density reached 0.5 to 1 (~6 h), washed once in water, and frozen. Luciferase activities were determined using firefly and *Renilla* luciferase assay systems (Promega). Cells were suspended in 250 μl CCRL buffer (*Renilla*) or buffer A (firefly) (25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 0.1% Triton X-100) supplemented with phenylmethylsulfonyl fluoride (PMSF) and lysed by being vortexed with glass beads three times for 1 min each with intermediate cooling. Cell debris was removed by centrifugation, 5 μl yeast extract was mixed with 20 μl (25 μl for *Renilla*) substrate working solutions, and bioluminescence was recorded in an InfinTe M200 microplate reader (Tecan) with an integration time of 1 s. Protein concentrations were determined in microplates by the Bradford method (Bio-Rad). Standard deviations were calculated from duplicate measurements of at least 4 independent cells. Reporter assays using green fluorescent protein (GFP) were carried out as described previously (20, 32).

Miscellaneous methods. The following published methods were used: manipulation of DNA and PCR (44), transformation of yeast cells (16), isolation of yeast mitochondria and postmitochondrial supernatant (7), immunostaining (19), Northern blotting (20), and determination of enzyme activities of isopropylmalate isomerase, Leu1, and aconitase (32); dihydroxy-acid dehydratase, Ilv3 (9); and ferrochelatase (3). Error bars represent the standard errors of the means (SEM) ($n \geq 4$).

RESULTS

No significant role of Aft1 in downregulation of iron-responsive genes. In order to analyze the mechanisms underlying the downregulation of iron-responsive genes upon iron deprivation, we analyzed the transcription of two representative genes involved in amino acid biosynthesis and respiration. *LEU1*, encoding the Fe/S protein isopropylmalate isomerase of the leucine biosynthesis pathway, was chosen because the gene displays the strongest downregulation upon iron starvation in several strain backgrounds (20, 37, 47). *CYC1*, encoding cytochrome *c*, was selected because it displays the strongest iron responsiveness of all genes involved in respiration (20, 37, 47). Northern blot analysis confirmed the downregulation of these genes after 16 h of cultivation under iron-limiting conditions, while *HMX1*, a member of the yeast iron regulon, was strongly induced (Fig. 1A and data not shown [20]). Upon depletion of two components of the mitochondrial ISC assembly and export systems, Yah1 and Atm1, transcription of both *LEU1* and *CYC1* was modified by a margin similar to that in iron-deprived wild-type cells, confirming the central role of the mitochondrial ISC systems in the regulation of cellular iron homeostasis (20, 43). Enzyme activities of Leu1 were more than 20-fold reduced upon iron deprivation, as expected from the loss of its Fe/S cluster, and yet protein levels of Leu1 were reduced only ~2-fold upon iron deprivation (Fig. 1B). Cytochrome *c* levels of mitochondria from iron-starved cells dropped below the level of detection (Fig. 1C).

Since under iron starvation conditions the iron-responsive transcription factor Aft1 is activated and the iron regulon, including the *CTH1* and *CTH2* genes, is induced, we tested whether Aft1 induction might be responsible for the observed decrease in *LEU1* and *CYC1* expression. In cells overproducing Aft1 (i.e., under constitutive induction of the iron regulon), Leu1 displayed wild-type activities and cytochrome *c* was present at wild-type levels under iron-replete conditions (Fig. 1B and C). A similar picture was observed for *HEM15* encoding ferrochelatase. While *HEM15* mRNA levels (20), protein levels, and ferrochelatase activities were reduced in iron-deprived yeast cells, they remained virtually unchanged in cells overproducing Aft1 (Fig. 1D). In contrast, mRNA levels of *FET3*, a member of the iron regulon, were strongly induced upon overproduction of Aft1 (Fig. 1E). These data suggest that the transcription factor Aft1 alone plays no significant role in the iron-responsive expression of *LEU1*, *CYC1*, or *HEM15*, despite an induction of the mRNA-degrading proteins Cth1 and Cth2. The fact that the overproduction of Aft1 is not associated with any obvious mitochondrial defects or with the downregulation of a significant number of genes in DNA microarray analyses confirms these observations (20, 42).

Dual regulation of the iron-dependent expression of *LEU1*. For a detailed analysis of *LEU1* gene expression, we used luciferase-based reporter assays. The 900-bp segment immediately upstream of the start codon of *LEU1* was inserted in front of the firefly luciferase gene of the yeast vector p416MET25-luc2 (32). Cell extracts of W303A wild-type cells harboring the resulting vector pLEU1-Luc2 displayed significant amounts of

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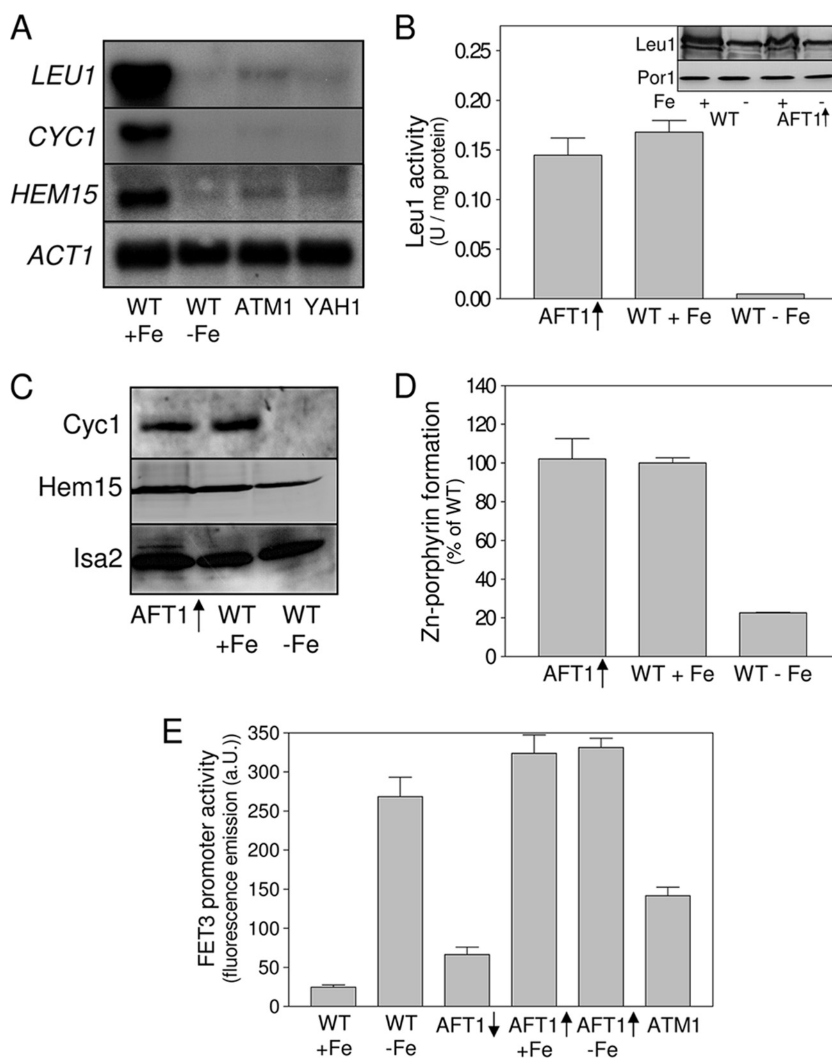

FIG. 1. Aft1 alone plays no significant role in the downregulation of *LEU1*, *CYC1*, and *HEM15*. (A) Northern blot analysis of *LEU1*, *CYC1*, and *HEM15* transcription. Total RNA was isolated from exponentially growing wild-type cells (WT) cultivated under iron-replete conditions in the presence of 50 μM $FeCl_3$ (+Fe) or under iron-deprived conditions in the presence of 50 μM bathophenanthroline for 16 h (−Fe) and from Atm1- or Yah1-depleted Gal-ATM1 (ATM1) or Gal-YAH1 (YAH1) cells grown under iron-replete conditions in synthetic complete minimal (SD) medium. RNA was separated on agarose gels, blotted onto nylon membranes, and hybridized with ^{32}P-labeled probes. *ACT1* served as a loading control (20). (B) Cell extracts of wild-type cells cultivated for 16 h in the presence of 50 μM $FeCl_3$ (+Fe) or 50 μM bathophenanthroline (−Fe) and wild-type cells overproducing Aft1p from vector p424MET3-AFT1 (AFT1↑) were analyzed for Leu1 activity. The inset shows an immunostaining for Leu1 and Por1 in wild-type and Aft1-overproducing cells cultivated under iron-replete and iron-deprived conditions. (C) Mitochondria were isolated from iron-replete, iron-starved, or Aft1-overproducing wild-type cells (AFT1↑). Protein levels of cytochrome *c* (Cyc1) and ferrochelatase (Hem15) were assessed by immunostaining. An immunostaining for Isa2 served as a loading control. (D) Ferrochelatase activities of mitochondria were determined by monitoring the insertion of Zn^{2+} into protoporphyrin IX. (E) The wild-type strain (W303A), the Atm1-depleted Gal-ATM1 strain (ATM1), and the W303 wild-type strain overproducing Aft1 from the methionine-regulated vector p424MET3-AFT1 harboring reporter plasmid pFET3-GFP were cultivated under iron-replete and iron-depleted conditions. Aft1-overproducing cells were cultivated under repressive conditions in the presence of methionine (AFT1↓) or under inducing conditions in the absence of methionine (AFT1↑). *FET3* promoter activities were determined by measuring the GFP-specific fluorescence emission of logarithmically grown cells. Error bars indicate the standard errors of the means ($n \geq 4$).

luciferase-based luminescence under iron-replete conditions, and these were reduced 10-fold upon cultivation under iron-limiting conditions in the presence of the iron chelator bathophenanthroline for 24 h (Fig. 2A). In cells depleted of components of the mitochondrial ISC assembly and export systems, Ssq1, Yah1, and Atm1, a ~4-fold reduction of luciferase activity was observed. These data are similar to those of the Northern blot analysis (Fig. 1A). Recent work has shown that terminators play an important role in iron-responsive gene expression in that they contain AU-rich elements (AREs) as binding sites for the RNA-binding proteins Cth1 and Cth2, which induce mRNA degradation under iron-limiting conditions (34, 37, 38). Corresponding potential AREs for Cth1 and Cth2 are present in the 3′ UTR of *LEU1* 90 and 110 bp downstream of the stop codon (not shown). In addition, the *CYC1* terminator present in vector pLEU1-Luc2 is involved in

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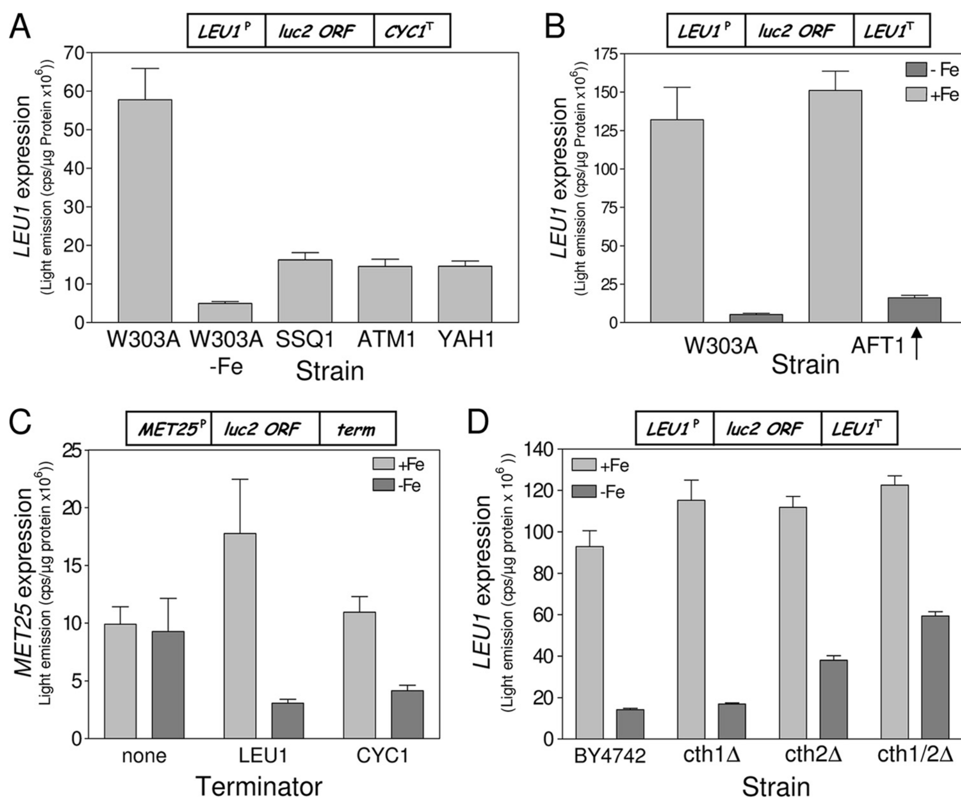

FIG. 2. Both promoter and terminator mediate the iron-responsive expression of *LEU1*. (A) W303A wild-type cells cultivated for 24 h in the presence of 50 μM $FeCl_3$ (+Fe) or 50 μM bathophenanthroline (−Fe) and Atm1-, Ssq1-, or Yah1-depleted Gal-ATM1, Gal-SSQ1, and Gal-YAH1 cells harboring reporter plasmid pLEU1-Luc2 (containing the *CYC1* terminator; see box on top) were grown to mid-log phase. The luciferase-derived luminescence of clarified cell extracts was quantified. (B) Wild-type (W303A) and Aft1-overproducing cells (AFT1 ↑) harboring reporter plasmid pLEU1/LEU1-term were cultivated for 24 h under iron-replete and iron-depleted conditions, and the luciferase activity of cell extracts was quantified. (C) *luc2* transcript levels from the *MET25* promoter under iron-replete and iron-limiting conditions were determined in wild-type (W303) cells. The reporter plasmids harboring the luciferase gene contained either no terminator (pMET25-Luc2, none), or the 3′ UTRs (term) of *LEU1* (pMET25/LEU1-term) or *CYC1* (p416MET25-luc2), respectively. (D) Wild-type (BY4742) and the respective *cth1*Δ, *cth2*Δ, and *cth1*Δ/*cth2*Δ (*cth1/2*Δ) deletion strains were transformed with the reporter plasmid pLEU1/LEU1-term. Cells were cultivated under iron-replete and iron-depleted conditions for 24 h, and *luc2* transcript levels were determined. Error bars indicate the SEM ($n \geq 4$). ORF, open reading frame.

Cth1/2-mediated mRNA decay (38). Replacement of the *CYC1* terminator in vector pLEU1-Luc2 by a 470-bp region immediately downstream of the *LEU1* stop codon (resulting in vector pLEU1/LEU1-term) gave a twofold increase in expression (compare Fig. 2A and B), and a strong, 25-fold downregulation was observed upon iron deprivation. No significant change in *LEU1* expression was observed upon overproduction of Aft1, confirming that this transcription factor plays no significant role in *LEU1* expression. The *LEU1* terminator conferred iron responsiveness on the *MET25* promoter, which shows no iron regulation *per se*, that was twofold stronger than that obtained with the terminator of *CYC1* (Fig. 2C). This demonstrates that the *LEU1* terminator is involved in iron-responsive gene expression. Finally, luciferase expression under the control of the *LEU1* promoter and terminator was studied in cells lacking the RNA-binding proteins Cth1 and Cth2 (Fig. 2D). Under iron-replete conditions, deletion of *CTH1* and *CTH2* had little effect. Under iron-limiting conditions, deletion of *CTH1* had little effect while that of *CTH2* increased *LEU1* expression by a factor of 2. In the *cth1*Δ/*cth2*Δ double deletion mutant, expression under iron-limiting conditions increased to almost 50% of the value under iron-replete conditions. On the one hand these data indicate that Cth1 and Cth2 cooperate in inducing the degradation of the *LEU1* mRNA upon iron deprivation. On the other hand, they show that Cth1 and Cth2 do not fully account for the decreased expression under iron-depleted conditions and hence point toward an important role of the *LEU1* promoter in this process. We therefore analyzed the reason for the iron dependence of the *LEU1* promoter in more detail.

Several genes of the biosynthesis pathway for the branched-chain amino acids leucine, isoleucine, and valine are under the control of the transcriptional activator Leu3 (25). A single Leu3 binding site is predicted between positions −175 and −230 upstream of the start codon of the *LEU1* open reading frame (Fig. 3A) (12, 13, 29, 30). Promoter deletions from the 5′ side up to −230 bp did not significantly affect *LEU1* expression, while deletions that removed the putative Leu3 binding site resulted in a collapse of expression to basal transcription levels (Fig. 3A). Deletion of *LEU3* results in a strong decline of *LEU1* expression, confirming that *LEU1* is under the dominant control of a single transcription factor, similarly to other genes

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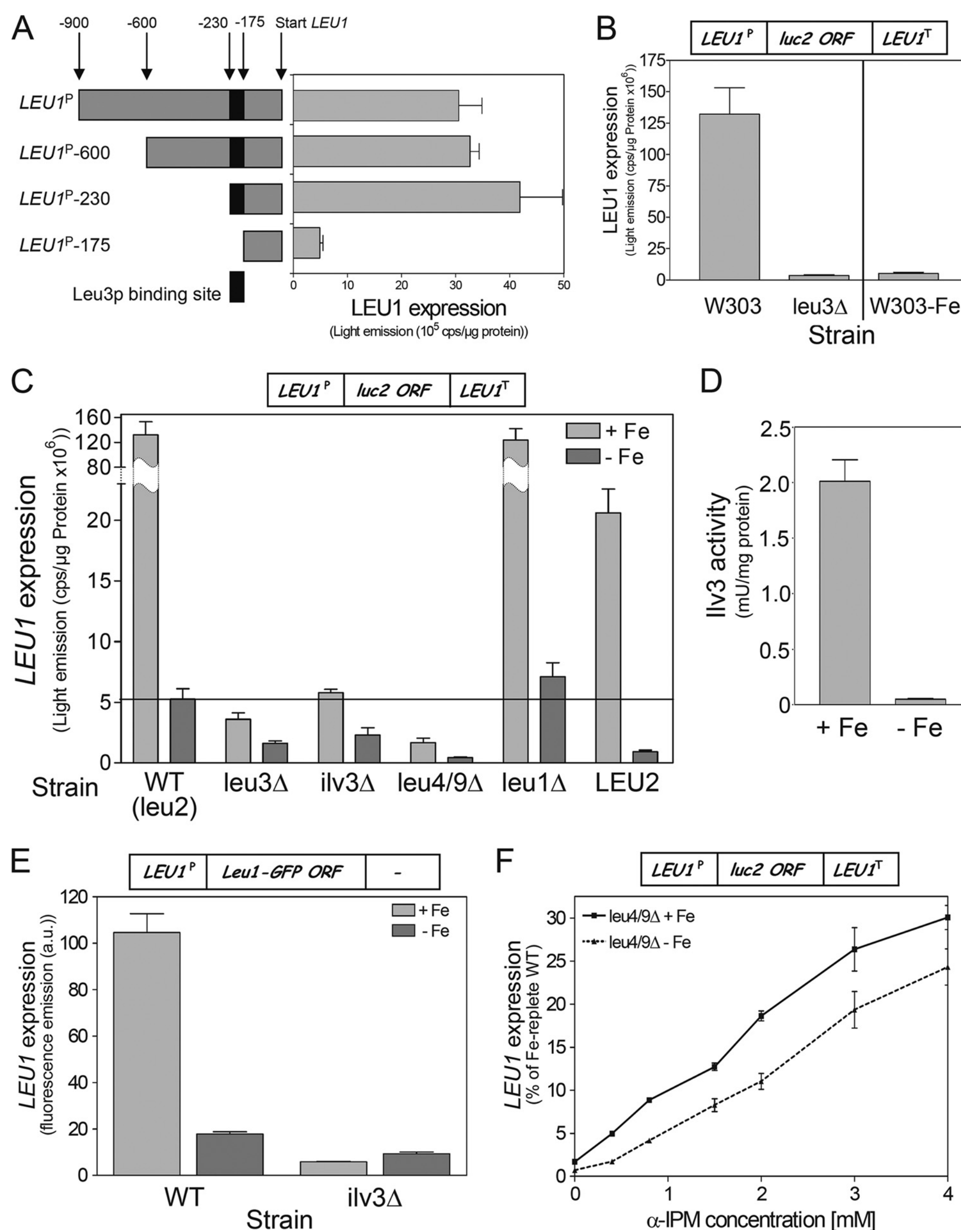

FIG. 3. Iron-responsive transcription of *LEU1* is mediated by cellular levels of α-isopropylmalate. (A) Wild-type cells (BY4742) were transformed with luciferase-based reporter plasmids harboring truncated versions of the *LEU1* promoter as indicated (left), and luciferase activities of cell extracts were quantified (right). (B) *LEU1* expression was determined in wild-type cells (W303) cultivated under iron-replete and iron-depleted (−Fe) conditions for 24 h and in the *leu3*Δ deletion strain (BY4742) harboring reporter plasmid pLEU1/LEU1-term (see box on top). (C) *LEU1* expression was determined in strains (W303) carrying deletions of the indicated genes of the biosynthetic pathway for leucine, isoleucine, and valine; a W303 strain harboring a wild-type *LEU2* allele (LEU2); and the *leu3*Δ strain (BY4742) (Fig. 4C). Cells were transformed with plasmid pLEU1/LEU1-term (box on top) and cultivated under iron-replete and iron-depleted conditions for 24 h. (D) Enzyme activities of the yeast dihydroxy-acid dehydratase Ilv3 were determined in mitochondria isolated under anaerobic conditions from wild-type cells cultivated under iron-replete and iron-depleted conditions. (F) Wild-type (BY4741) and *ilv3*Δ cells harboring a genomic copy of a terminatorless *LEU1* gene with a C-terminally fused GFP were cultivated under iron-replete and iron-depleted conditions for 24 h. Promoter activities were determined by measuring the GFP-specific fluorescence emission of logarithmically grown cells. (F) *LEU1* expression was determined in *leu4/9*Δ cells cultivated in SD medium supplemented with increasing amounts of α-IPM under iron-replete or iron-depleted conditions. Cells contained reporter plasmid pLEU1/LEU1-term. Error bars indicate the SEM ($n \geq 4$). ORF, open reading frame; WT, wild type.

of the branched-chain amino acid biosynthetic pathway (Fig. 3B) (13, 25). Moreover, remnant *LEU1* expression levels in *leu3*Δ cells were in the same range as those found in iron-deprived wild-type cells (Fig. 3B). These results indicate that the lack of another metabolite attenuates the ability of Leu3 to activate LEU1 upon iron deficiency.

The Leu3 transcription factor requires α-isopropylmalate (α-IPM), a metabolic intermediate of the branched-chain

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amino acid biosynthesis and the substrate of Leu1, as a transcriptional coactivator (25, 49). In order to study the effect of α-IPM on *LEU1* expression, we took advantage of strains carrying deletions in individual genes of the branched-chain amino acid biosynthesis pathway. Both Ilv3 and Leu4/9 enzymes are required for the production of α-IPM in this pathway (see Fig. 4C). In *ilv3*Δ and *leu4/9*Δ cells *LEU1* expression was reduced to basal levels similar to those seen in *leu3*Δ and iron-deprived wild-type cells (Fig. 3C). In addition, the iron responsiveness of *LEU1* was strongly reduced in these cells. Deletion of *LEU1*, which lies downstream of α-IPM synthesis in the pathway for leucine, showed wild-type expression from the pLEU1/LEU1-term plasmid. *LEU2* is inactivated in virtually all common yeast laboratory strains. Complementation of the *leu2* mutation in W303 wild-type cells with a wild-type genomic *LEU2* copy reduced *LEU1* expression under iron-replete conditions ~9-fold, presumably by restoring the metabolic flux through the pathway and thus decreasing the levels of α-IPM (see Fig. 4C). Iron deprivation of *LEU2* cells reduced *LEU1* mRNA levels ~22-fold, which is very close to the levels observed in the *leu2* background of our W303 wild-type cells. Apparently, the *leu2* background of common yeast laboratory strains amplifies the expression of *LEU1* without significantly changing its iron-responsive regulation. Taken together, these observations demonstrate a central activating role of α-IPM for *LEU1* expression and are consistent with earlier data for other genes of branched-chain amino acid biosynthesis (25).

Branched-chain amino acid biosynthesis contains two iron-dependent enzymes, the Fe/S proteins Ilv3 (mitochondrial dihydroxy-acid dehydratase) and Leu1 (9, 25) (Fig. 4C). Similarly to Leu1 (Fig. 1B), the activity of Ilv3 was virtually undetectable in wild-type cells upon iron depletion (Fig. 3D). Thus, an iron-starved wild-type cell likely contains low levels of α-IPM and in this respect behaves similarly to an *ilv3*Δ strain. This conclusion easily explains why *LEU1* expression levels in iron-deprived cells are similar to those of mutants that are impaired in the production of α-IPM or that lack the transcription activator Leu3 (Fig. 3C). In order to verify the iron-responsive transcription of *LEU1* independently of the *LEU1* terminator, we took advantage of a yeast strain that contains a chromosomal *LEU1-GFP* gene fusion replacing the *LEU1* terminator region with an in-frame *GFP/HIS3* cassette. This strain was previously used in a systematic protein localization study with *S. cerevisiae* (22). The GFP-specific fluorescence of this strain was ~6-fold reduced upon cultivation under iron-deprived conditions below that in iron-replete conditions (Fig. 3E). Upon deletion of *ILV3* in this strain, the fluorescence emission strongly declined to basal levels similar to those under iron deprivation and showed virtually no iron response. Finally, in order to directly show the regulatory role of α-IPM in *LEU1* expression under iron-limiting conditions, α-IPM was added to *leu4/9*Δ cells that are unable to synthesize α-IPM (Fig. 4C). Under both iron-replete and iron-depleted conditions, added α-IPM increased *LEU1* expression in virtually identical concentration-dependent fashions that were almost linear up to the maximal tested amount of the inducer (Fig. 3F). We therefore conclude that the iron-dependent synthesis of α-IPM fully explains the iron-responsive transcription of *LEU1*.

Transcription, however, was not alone responsible for the iron-dependent modulation of *LEU1*, since a small (~4-fold) iron response was retained in cells that are unable to activate the *LEU1* promoter via α-IPM and Leu3 (i.e., in *ilv3*Δ, *leu4/9*Δ, and *leu3*Δ cells) (Fig. 3C and 4A). Likely, this residual iron response was conferred by the *LEU1* terminator alone. If so, this response should be similar to that of a truncated *LEU1* gene that cannot be activated by Leu3. In order to test this conclusion, we took advantage of a *LEU1* core promoter construct, pLEU1^P-175/LEU1term, which lacks the Leu3 activator domain (Fig. 3A). Indeed, iron depletion reduced the expression of the *LEU1* core promoter construct in wild-type cells by virtually the same margin (~4-fold) as that for the full-length *LEU1* promoter in *ilv3*Δ and *leu4/9*Δ cells (Fig. 4A). This reduction was quantitatively similar to that observed for the iron-indifferent *MET25* promoter upon insertion of the *LEU1* terminator (5.8-fold) (Fig. 2C). In addition, deletion of *CTH2* completely abolished the iron responsiveness of the *LEU1* core promoter construct (Fig. 4B). In the *cth1*Δ/*cth2*Δ double mutant, expression was even higher upon iron deprivation than under iron-replete conditions. Most likely, the residual iron response of *LEU1* in cells that are unable to activate *LEU1* transcription is quantitatively conferred by Cth1-Cth2 function in *LEU1* mRNA degradation. Clearly, the iron-responsive expression pattern of the *LEU1* core promoter in cells lacking Cth1 and/or Cth2 differed from that of the full promoter construct (Fig. 2D).

Taken together, these data show that the iron-responsive expression of *LEU1* can be quantitatively explained by a combined regulation through both the promoter and the terminator (Fig. 4C). *LEU1* transcription is controlled by the activity of Leu3, which is modulated by cellular levels of α-IPM. These are low under iron-limiting conditions due to the inactivation of the Fe/S protein Ilv3, which is essential for α-IPM production. The *LEU1* terminator contains information for posttranscriptional mRNA degradation facilitated by the two mRNA-binding proteins Cth1 and Cth2 (53). Quantitatively, transcriptional regulation contributes about sixfold (Fig. 3E) and posttranscriptional mRNA decay contributes fourfold to the iron responsiveness of *LEU1*, respectively (Fig. 4A). Assuming that both mechanisms are cumulative, a 24-fold downregulation of *LEU1* upon iron deprivation is expected, which is close to the experimental data (25-fold effect in Fig. 2B). Thus, the combination of the two regulatory mechanisms of metabolite-linked iron-responsive transcription and posttranscriptional mRNA degradation provides a quantitative description of the iron-responsive expression of *LEU1*.

Hap-mediated iron-dependent expression of *CYC1*. The above data show that the iron-dependent remodeling of levels of a key regulatory molecule of a biochemical pathway confers an iron-responsive gene expression pattern. In *S. cerevisiae*, the iron-containing cofactor heme functions as a key regulatory metabolite during the adaptation to hypoxic conditions (6, 55, 56). Since heme levels are low in iron-deprived cells, it was tempting to speculate whether heme also functions as a regulatory molecule in iron-responsive expression of genes involved in respiration. In order to explore this possibility, we analyzed the *CYC1* gene encoding the heme protein cytochrome *c*, which is transcribed in an iron-responsive manner (36). Apart from the roles of Cth1 and Cth2 in *CYC1* mRNA degradation, little is known concerning the mechanism of the iron responsiveness of *CYC1* (38). The *CYC1* promoter harbors two up-

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FIG. 4. Dual regulation of the iron responsiveness of *LEU1*. (A) The ratio of *LEU1* expression under iron-replete conditions to that under iron-depleted conditions in the indicated strains as derived from Fig. 3C (right panel) was compared with that of a *LEU1* core promoter construct (pLEU1^{P}-175/LEU1-term) in W303 wild-type cells. (B) Expression levels of the *LEU1* core promoter were determined under iron-replete (+Fe) and iron-depleted (−Fe) conditions in wild-type cells (BY4742) and the *cth1*Δ, *cth2*Δ, and *cth1*Δ/*cth2*Δ (*cth1*/*2*Δ) deletion strains harboring reporter plasmid pLEU1^{P}-175/LEU1-term (box on top). (C) Model for the iron-responsive expression of *LEU1* in *S. cerevisiae*. Abbreviations: α,β-DIV, α,β-dihydroxyisovalerate; α-KIV, α-ketoisovalerate; β-IPM, β-isopropylmalate; α-KIC, α-ketoisocaproate. WT, wild type; ORF, open reading frame.

stream activating sequences, UAS1 and UAS2, which are controlled by the transcriptional activator Hap1 and the Hap2-5 complex (17) (Fig. 5A). This tandem structure is shared by other genes involved in respiration (40, 45). While the activating molecule of the Hap2-5 complex is unknown, Hap1 requires heme for transcriptional activity (6, 55, 56).

In order to quantify the contribution of the *CYC1* terminator to the iron-responsive expression of *CYC1*, we employed a luciferase reporter construct, pΔUAS1/2-hRluc, which lacks the two upstream activating domains (Fig. 5A). The analysis was carried out in raffinose-containing medium in order to avoid glucose repression. In BY4742 wild-type and *cth1*Δ cells, only a 1.3-fold-reduced expression of pΔUAS1/2-hRluc was observed upon iron deprivation (Fig. 5B). This effect was surprisingly small, as the *CYC1* terminator is a known target of both Cth proteins (38). In *cth2*Δ cells, the expression of pΔUAS1/2-hRluc was twofold higher under iron-limiting conditions than under iron-replete conditions and increased to >3-fold in *cth1*Δ/*cth2*Δ cells. Higher expression under iron-limiting than under iron-replete conditions in *cth1*Δ/*cth2*Δ cells seems to be a common theme for core promoters of ARE-containing genes, as this effect was also observed for *LEU1*

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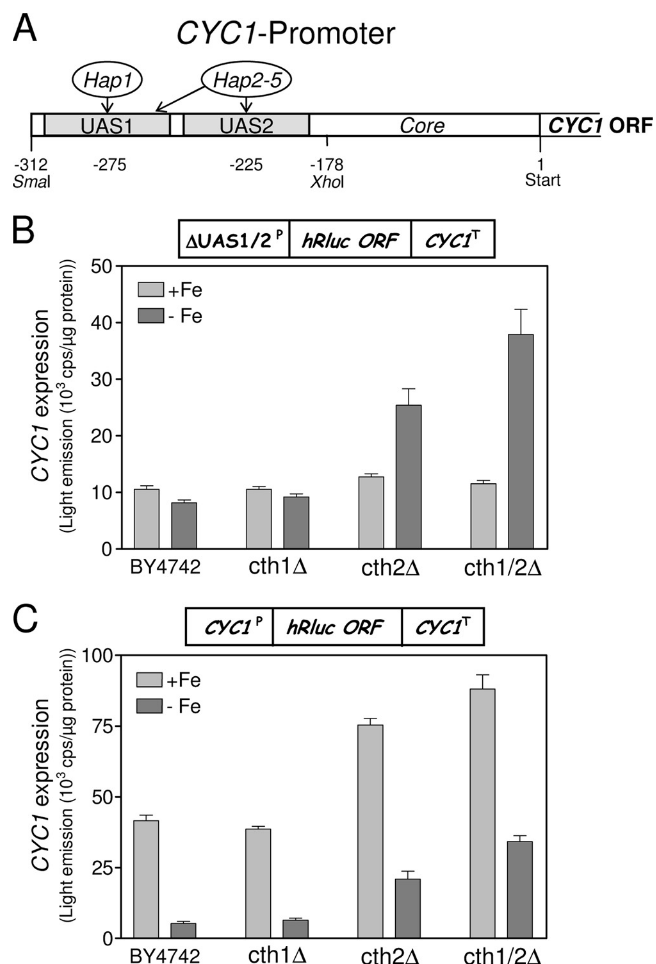

FIG. 5. The iron responsiveness of *CYC1* is mediated by transcription and Cth1/Cth2-dependent mRNA degradation. (A) Model for the *CYC1* promoter (17). (B and C) The wild-type strain (BY4742) and the *cth1*Δ, *cth2*Δ, and *cth1*Δ/*cth2*Δ (*cth1*/*2*Δ) isogenic deletion strains were transformed with the reporter plasmid pΔUAS1/2-hRluc (B) or pCYC1-hRluc (C) (boxes on top). Cells were cultivated under iron-replete (+Fe) and iron-depleted (−Fe) conditions in the presence of raffinose for 24 h, and the *Renilla* luciferase level was determined in cell extracts. Error bars indicate the SEM ($n \geq 4$). ORF, open reading frame.

(Fig. 4B). Most likely, this induced expression reflects an increased mRNA stability that allows an increased number of translations in *cth1*Δ/*cth2*Δ cells. A promoter construct, pCYC1-hRluc, harboring the complete *CYC1* core promoter, including the two upstream activation sequence (UAS) regions (Fig. 5A), showed a fourfold-higher expression level in BY4742 wild-type cells than did the activatorless core promoter construct pΔUAS1/2-hRluc (Fig. 5C). In addition, a ~6-fold downregulation was observed for the full *CYC1* promoter construct upon iron deprivation of wild-type cells, in contrast to the weak effect seen with pΔUAS1/2-hRluc. Thus, the promoter appears to be the dominant element for the iron-responsive expression of *CYC1*. In *cth1*Δ cells *CYC1* was expressed similarly to wild-type cells, while the deletion of *CTH2* alone or in combination with *CTH1* increased the expression under iron-replete conditions and yet attenuated the downregulation of *CYC1* under iron-limiting conditions. The fact that the deletion of *CTH2* induced a stronger deregulation under iron deprivation of all constructs tested than did that of *CTH1* confirms the dominant role of Cth2 in posttranscriptional mRNA decay upon long-term iron deprivation (38). Taken together, these data show that the iron responsiveness of *CYC1* is explained by a combination of transcriptional effects involving promoter elements and by mRNA degradation via the Cth proteins. The former mechanism is the dominant effect.

We attempted to identify the reason for the iron responsiveness of *CYC1* transcription by analyzing the roles of the two activators Hap1 and Hap2-5. The iron responsiveness of the *CYC1* promoter was confined to the two upstream activating domains UAS1 and UAS2, as a construct harboring the 1-kb 5′ upstream region of the *CYC1* open reading frame displayed an expression pattern in wild-type cells almost identical to that of the reporter construct pCYC1-hRluc above, which included only UAS1 and UAS2 (not shown). We therefore concentrated our analysis on these two promoter regions and, in addition to wild-type cells, employed cells in which *HAP1*, *HAP2*, or *HAP4* was deleted or *HAP4* was overexpressed (Fig. 6A and B). The extent of remodeling between iron-depleted and iron-replete conditions is presented in Fig. 6C. Removal of UAS1 and that of UAS2 reduced *CYC1* expression in BY4742 wild-type cells under iron-replete conditions by similar margins but did not significantly affect the expression level upon iron deprivation (Fig. 6A, top panel). Thus, similarly to *LEU1*, a reduced transcriptional activation of *CYC1* goes along with a diminished iron responsiveness. We then studied the role of Hap1 in iron-responsive transcription of *CYC1* which binds to the UAS1 region (6, 18, 55, 56). Most laboratory yeast strains harbor a transposon insertion at the *HAP1* locus that replaces the C-terminal 12 amino acids with a 30-amino-acid random sequence (14). In BY4742, *CYC1* expression dropped fourfold upon complete deletion of the *HAP1* open reading frame (Fig. 6A, middle panel). A similar effect was seen in the W303 background (not shown), confirming previous findings that indicate that the natural *hap1* mutant of S288c-derived strains retains at least limited function (14). Iron deprivation of BY4742 *hap1*Δ cells reduced *CYC1* expression by a factor of 2.5, which is >2-fold less than that in the wild type (Fig. 6A, middle panel). Iron responsiveness of *CYC1* was reduced further in *hap1*Δ cells (to 1.7-fold) upon removal of the first activating domain, UAS1 (Fig. 6A, middle panel). Strikingly, a reporter construct, pΔUAS2-hRluc, which lacks the second activation domain of *CYC1*, which is not targeted by Hap1, displayed virtually no iron-dependent expression in *hap1*Δ cells (Fig. 6A, middle panel, and 6C). This observation clearly demonstrates that the Hap1 transcription factor contributes significantly to the iron-responsive expression of *CYC1*. Likely, the impact of Hap1 in this process is much stronger in strains that harbor a wild-type *HAP1* allele.

Finally, we analyzed whether the inactivation of the Hap2-5 complex, the second activator that binds predominantly to the UAS2 activator element, influences the iron-responsive expression of *CYC1* (11, 31). In *hap2*Δ cells that lack the core component of the Hap2-5 complex, *CYC1* expression was twofold lower than that in wild-type cells and also displayed a 2.5-fold-lower iron response (Fig. 6A, bottom panel). In addition, the iron response of the pΔUAS1-hRluc construct was

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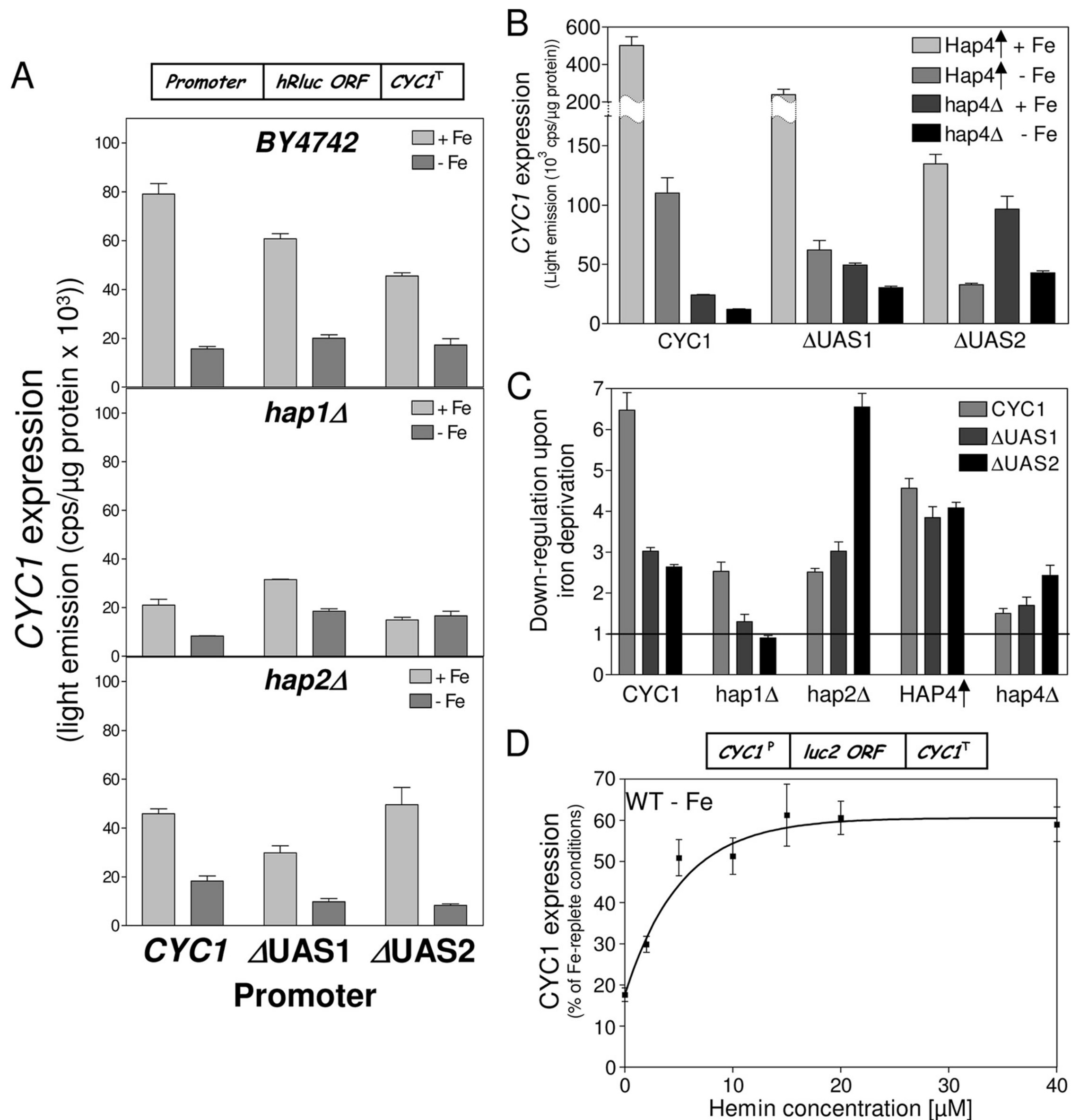

FIG. 6. Hap1 and Hap4 are involved in the iron-responsive expression of *CYC1*. (A) Wild-type (BY4742) cells and the *hap1*Δ and *hap2*Δ isogenic deletion strains were transformed with reporter plasmid pCYC1-hRluc, pΔUAS1-hRluc, or pΔUAS2-hRluc carrying the indicated *CYC1* promoters (Fig. 5A). Cells were cultivated under iron-replete (+Fe) and iron-depleted (−Fe) conditions for 24 h, and the *Renilla* luciferase-derived luminescence was determined. (B) Expression of the different *CYC1* promoter constructs was determined in *hap4*Δ and wild-type cells overproducing Hap4 (Hap4 ↑) as described for panel A. (C) The ratio of the iron-responsive expression was calculated from the quotas of the expression of the different *CYC1* promoter constructs under iron-replete and iron-deprived conditions for the indicated strains in panels A and B. (D) Wild-type cells expressing the *C. elegans* heme importer CeHRG4, which harbored the reporter plasmid pCYC1-Luc2, were cultivated under iron-depleting conditions and supplemented with increasing amounts of hemin for 16 h, and the luciferase-derived luminescence was determined. Error bars indicate the SEM ($n \geq 4$). ORF, open reading frame.

also ~2-fold lower in *hap2*Δ cells than in wild-type cells, demonstrating that the Hap2-5 complex is contributing to the iron response of *CYC1*. However, the fact that the pΔUAS1-hRluc construct that is not regulated by Hap1 retained an iron response in *hap2*Δ cells at all indicates that further unknown transcription factors contribute to the iron responsiveness of *CYC1* that is conferred via UAS2. Remarkably, for the pΔUAS2-hRluc construct, an almost-wild-type (sixfold) down-regulation was observed in *hap2*Δ cells upon iron limitation (Fig. 6A, bottom panel, and 6C). This observation that a truncated *CYC1* promoter construct that is activated only by Hap1 via UAS1 displayed an iron response similar to that of the full *CYC1* promoter confirms the prominent role of Hap1 in this process. The wild-type iron responsiveness of the pΔUAS2-hRluc construct was not observed in *hap4*Δ cells that lack the principal activator component of the Hap2-5 complex, suggesting that an inactivated Hap2-5 complex occupies the promoter and by this interferes with the activation via Hap1. Neverthe-

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less, both expression and iron responsiveness of the full *CYC1* promoter were reduced by similar margins in *hap4*Δ and *hap1*Δ cells (Fig. 6B and C). This suggests that the two independent Hap activator systems contribute equally to global expression and iron responsiveness of *CYC1*. Overproduction of Hap4 increased *CYC1* expression 20-fold over that in *hap4*Δ cells, as expected. Expression of the pΔUAS1-hRluc construct was increased 4.8-fold and that of pΔUAS2-hRluc was increased 1.4-fold over that in *hap4*Δ cells. At the same time, the iron response of the full *CYC1* promoter also increased from 2-fold in *hap4*Δ cells to 4.5-fold in Hap4-overproducing cells, confirming the involvement of the Hap2-5 complex in the iron regulation of *CYC1*. Hap4 overproduction also increased the iron responsiveness of the pΔUAS1-hRluc construct 2.2-fold over that in *hap4*Δ cells and that of pΔUAS2-hRluc 1.6-fold (Fig. 6B and C). Thus, the effect of Hap4 on the iron responsiveness of *CYC1* is transmitted via both activating domain UAS2 and, to a lesser extent, UAS1. The fact that this Hap4-dependent modulation was mediated via both upstream activating domains is in accordance with previous findings (28).

Taken together, these data demonstrate that the transcriptional activators Hap1 and Hap4 play decisive roles for the iron-responsive expression of *CYC1*. The heme-dependent activation of Hap1 likely explains how Hap1 contributes to the downregulation of *CYC1* upon iron limitation. Heme, an iron-containing cofactor, is sparse under conditions of iron deprivation. At the same time, the activity of ferrochelatase, the enzyme that catalyzes the last step in heme synthesis, is significantly reduced under these conditions (Fig. 1D). Under our experimental conditions, heme levels were ~20-fold reduced in wild-type cells cultivated in the presence of bathophenanthroline (not shown). In order to directly show the involvement of heme in transcriptional adaptation to iron-deprived conditions, hemin was added to iron-deprived wild-type cells that expressed the heme importer CeHRG4 from *Caenorhabditis elegans* (39). Hemin increased *CYC1* expression in a concentration-dependent fashion up to a maximum of ~60% of that observed under iron-replete conditions (Fig. 6D). No significant induction was seen when hemin was added to cells cultivated under iron-replete conditions (data not shown). In addition, the activity of aconitase, an Fe/S protein, increased only marginally in iron-deprived cells upon feeding with hemin, indicating that the iron that is imported into the cells by heme was not converted into a biologically available form in large amounts (data not shown). The observed induction of *CYC1* was therefore mainly due to increasing levels of intracellular hemin. Thus, the lack of activation of Hap1 due to diminishing levels of intracellular heme plays a decisive role in the transcriptional adaptation of respiratory genes to iron-deprived conditions.

DISCUSSION

S. cerevisiae responds to iron deprivation with a massive adaptive remodeling of mRNA transcript levels that includes genes for components of mitochondrial respiration and the citric acid cycle and genes involved in the biosynthetic pathways for ergosterol and unsaturated fatty acids, biotin, purines, and several amino acids (20, 24, 35, 37, 38, 47). The majority of genes that are downregulated upon iron deprivation are members of metabolic processes that contain iron-dependent enzymes. It was thus suggested that yeast adapts to iron deprivation by minimizing dispensable iron-dependent processes in order to liberate and spare iron for more-essential tasks (24, 35, 53). The mechanisms underlying this iron sparing, however, are not fully understood. In this work, we have studied the contribution of transcription to the iron responsiveness of two representative genes, *LEU1* and *CYC1*, which are strongly downregulated upon iron deprivation. Previous work focused on the central role of posttranscriptional mRNA degradation of iron-responsive genes upon iron deprivation involving the mRNA-binding proteins Cth1 and Cth2 (37, 38). Our current analysis identifies a novel mechanism of transcriptional regulation of iron-responsive genes that involves iron-dependent metabolites. This regulatory mechanism provides a second level of control in the adaptation of *S. cerevisiae* to iron-deprived conditions that operates in addition to the previously reported posttranscriptional mechanism. The sum of the two pathways allows the quantitative description of iron-responsive gene expression.

The biosynthetic pathway for branched-chain amino acids serves as an excellent example of a pathway that is subjected to iron sparing, as it contains two Fe/S proteins, isopropylmalate-isomerase (Leu1) and dihydroxy-acid dehydratase (Ilv3), that have no other known function outside the biosynthesis of isoleucine, leucine, and valine. (25). Leu1 is the most abundant Fe/S protein in *S. cerevisiae* (15), and consequently, *LEU1* displays the strongest downregulation upon iron starvation of all genes in several strain backgrounds (20, 37). Other iron-responsive genes of this pathway include *LEU2*, *BAT1*, *BAT2*, *ILV3*, *ILV5*, and *BAP2* (20, 37, 38, 47). The transcriptional activator Leu3 plays a central role in gene expression of the branched-chain amino acid biosynthesis, and several genes that are regulated by Leu3 are also iron responsive, including *GDH1*, encoding glutamate dehydrogenase involved in nitrogen assimilation (21, 25, 47). Leu3 is activated by the metabolite α-isopropylmalate, an intermediate of this pathway and the substrate of Leu1 (25, 49). Our data show that *LEU1* transcription is dominantly regulated by Leu3 and that *LEU1* expression in iron-starved wild-type cells is reduced to basal levels that are similar to those of *leu3*Δ cells or cells that are unable to synthesize α-IPM. In addition, *LEU1* displays only limited iron responsiveness in cells lacking Leu3. These observations identify Leu3 as an indirect iron-responsive transcription factor. The mechanism of the iron regulation via Leu3 is intimately linked to its activation by α-IPM. Under iron-deprived conditions, the Fe/S protein Ilv3, which is crucial for α-IPM production (25), is inactive due to the lack of its cofactor and the *ILV3* mRNA is the target of the Cth proteins (38). Thus, the downregulation of Leu3-dependent genes under iron deprivation is achieved through the back door via the iron-dependent modulation of cellular levels of a key regulatory metabolic intermediate, α-IPM, thus linking iron availability to metabolism (25).

In a similar fashion, the expression of lysine biosynthesis genes in *S. cerevisiae* depends on the inducer α-aminoadipate semialdehyde, a metabolic intermediate that activates a single transcription factor, Lys14 (8). Its synthesis essentially requires the Fe/S enzymes aconitase and homoaconitase, which operate early in the pathway. Further examples of biosynthetic path-

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ways that include both iron-dependent enzymes and iron-responsive genes are those for methionine (Met3), glutamate (Aco1 and Glt1), ergosterol (Erg3, Erg35, and Ncp1), and ubiquinone (Coq7). More indirect examples include pathways with enzymes containing lipoate or biotin, cofactors whose synthesis depends on the Fe/S proteins Lip5 and Bio2, respectively (10). For example, the genes of the glycine cleavage complex, a lipoate-containing enzyme, are downregulated upon iron deprivation (47). The common theme of all these examples is the inactivation of iron-containing enzymes. This global inactivation is seen upon iron deprivation and upon defects in mitochondrial Fe/S protein maturation. Thus, transcriptional effects caused by the inactivation of iron-containing enzymes likely contribute to the significant overlap in the transcriptional response of yeast cells to these two situations (2, 20).

Most genes that are downregulated upon iron deprivation are targets of the mRNA-binding proteins Cth1 and Cth2, which promote mRNA degradation upon iron limitation (34, 37, 38, 53). Nevertheless, the effects of Cth1 and Cth2 are too small to quantitatively explain the adaptation to iron deprivation. In addition, Cth1- and Cth2-dependent mRNAs are not significantly downregulated upon activation of Aft1, despite the fact that *CTH1* and *CTH2* are strongly induced in Aft1-up cells or upon Aft1 overproduction (42). Consequently, cells overproducing Aft1 show little biochemical evidence for the massive metabolic remodeling that is taking place upon iron starvation, except for the induction of the genes of the yeast iron regulon (20, 42, 47). As shown here, *LEU1* contains all the hallmarks of a gene targeted by the Cth proteins. Its 3′ downstream region contains potential binding sites for Cth1 and Cth2 and confers iron responsiveness on an iron-indifferent promoter. Consequently, *LEU1* mRNA accumulates in cells lacking *CTH1* and *CTH2* in a cooperative manner (38). The role of the terminator in the iron responsiveness of *LEU1* becomes evident in cells that are unable to activate Leu3 or with *LEU1* core promoter constructs that lack the Leu3 activating domain. These effects are relatively small (~4-fold), consistent with the systematic studies of Cth1 and Cth2 (37, 38). Yeast mutants lacking the *LEU1* terminator retain a strong iron-responsive *LEU1* expression that is completely lost by inactivating Leu3. Thus, in the absence of a terminator, the iron responsiveness of *LEU1* expression is conferred exclusively by Leu3-dependent transcription. Vice versa, the Cth proteins dominate the iron-regulated expression of *LEU1* when Leu3 is inactive. We conclude that α-IPM–Leu3-dependent transcription of *LEU1* adds a second important level of iron-responsive regulation, and only the combination of the effects conferred by both transcriptional and posttranscriptional regulation provides a quantitative portrayal of the iron responsiveness of *LEU1*.

CYC1, encoding cytochrome *c*, is a further example of an iron-responsive gene that is subjected to dual iron regulation by transcriptional and posttranscriptional mechanisms. Several metabolic processes that involve heme-containing enzymes are repressed upon iron deprivation, including respiration (20, 37, 38, 47). In *S. cerevisiae*, heme is used as a sensor for oxygen and functions both as a coactivator and, occasionally, as a transcriptional repressor (5, 27, 55, 56). Our observation that *CYC1* expression can be induced under iron-limiting conditions by feeding hemin demonstrates the crucial role of this cofactor for the iron responsiveness of heme-containing pathways, as suggested previously (24, 35). In *S. cerevisiae*, *CYC1* expression is dominated by the transcriptional activator Hap1 and the Hap2-5 complex (11, 17, 46, 55). This dual regulation is shared by several other genes involved in respiration (40, 45, 55). We show that both activator systems contribute significantly to the iron responsiveness of *CYC1*. The heme-activated transcription factor Hap1 plays an important role in the adaptation of *S. cerevisiae* to hypoxia. It is therefore no surprise that most genes of the Hap1 regulon are repressed under both hypoxic and iron-deprived conditions, two conditions that result in low levels of heme (1, 37, 50, 55, 56). In this context, in cells depleted of components of the mitochondrial ISC assembly systems, many genes of the Hap1 regulon are repressed, and yet surprisingly *HAP1* itself is induced (20). The fact that these cells are heme deficient underscores the finding that low heme levels are the primary physiological signal that provides the connection between the downregulation of genes of the Hap1 regulon upon hypoxia or iron deprivation and that in cells with defective mitochondrial ISC systems.

Taken together, in *S. cerevisiae* the regulation of those iron-responsive genes that are downregulated upon iron deprivation is conferred by two independent and likely additive mechanisms: transcriptional regulation and Cth1-Cth2-dependent posttranscriptional mRNA degradation. On the transcriptional level, the iron-responsive expression is achieved through the back door via key regulatory metabolites that are involved in activation of central transcription factors rather than directly by iron (25). Upon iron deprivation, the levels of these metabolites are reduced due to the inactivation of iron-dependent enzymes required for their biosynthesis or, in the case of heme, due to the lack of iron. The low levels of iron-dependent metabolites are then directly interpreted through diminished transcriptional activation. This indirect iron-responsive regulation likely operates in most pathways involving iron-dependent enzymes and thus easily explains the strong overlap of the transcriptomes of iron-deprived yeast cells and yeast with defects in the maturation of cellular Fe/S proteins (20). In addition, it leaves little room for additional transcription factors that sense iron by a direct mechanism similar to the Aft1 transcription factor in *S. cerevisiae*.

ACKNOWLEDGMENTS

We thank T. Mahnke and U. Koert for providing 2-3-dihydroxy-3-methylbutyrate.

We dedicate this paper to G. Kohlhaw.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 593, Gottfried-Wilhelm Leibniz program, and GRK 1216), von Behring-Röntgen Stiftung, Max-Planck Gesellschaft, and Fonds der chemischen Industrie.

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Supplement to Ihrig et al.

Supplementary Table I: Yeast Strains Used in This Study

Strain	Genotype	Method of Generation	Source/Reference
W303-1A	*MATa; ura3-1; ade2-1; trp1-1; his3-11,15; leu2-3,112*		(10)
W303-1B	*MATa; ura3-1; ade2-1; trp1-1; his3-11,15; leu2-3,112*		(10)
BY4741	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	obtained from Euroscarf	(1)
BY4742	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	obtained from Euroscarf	(1)
Gal-ATM1	W303-1B, *pATM1::pGAL1-10-LEU2*	gene replacement (pYEP51-Gal-ATM1)	(7)
Gal-YAH1	W303-1B, *pYAH1::pGAL1-10-LEU2*	gene replacement (pYEP51-Gal-YAH1)	(8)
Gal-SSQ1	W303-1A, *pSSQ1::pGAL1-10-HIS3*	PCR fragment (pFA6a-HIS3-Gal)	(11)
cth1Δ	BY4742*, cth1::HIS3*	PCR Fragment (pFA6a-HIS3MX6)	this work
cth2Δ	BY4742, *cth2::kanMX6;*	PCR fragment (pFA6a-KanMX4)	Euroscarf
cth1/2Δ	BY4742*, cth1::hphNT1; cth2::kanMX6*	PCR Fragment (pFA6a-hphNT1) (6)	this work
leu1Δ	W303-1A, *leu1::HIS3*	PCR fragment (pFA6a-HIS3)	this work
ilv3Δ	W303-1A, *ilv3::HIS3*	PCR fragment (pFA6a-HIS3)	this work
leu3Δ	BY4742, *cth2::kanMX6*		Euroscarf
leu4/9Δ	W303-A, *leu4::kanMX6; leu9::HIS3*	PCR fragments (pFA6a-HIS3 & pFA6a-KanMX4))	this work
LEU1-GFP	BY4741, *YGL009C-GFP::HIS3*		(5)
LEU1-GFP /ilv3Δ	*YGL009C-GFP, ilv3:: hphNT1*	PCR Fragment (pFA6a-hphNT1) (6)	this work
LEU2	W303-1A, *leu2-3::LEU2*	Integrative *LEU2* plasmid pBSC-HIS3/LEU2	this work
hap1Δ	BY4742, *hap1::natNT2*	PCR Fragment (pFA6a-natNT2)	this work
hap2Δ	BY4742, *hap2::kanMX6*		Euroscarf
hap4Δ	BY4742, *hap4::HIS3*	PCR fragment (pFA6a-HIS3)	this work

Gene disruptions and promoter exchanges were generated by PCR-based gene replacement and verified by PCR as described previously (12).

Supplementary Table II: Plasmid Constructs Used in This Study.

Plasmid	ORF	Promoter	Terminator	backbone	Source/Reference
p424MET3-AFT1	*AFT1-TAP-Tag*			p424MET3	(4).
p426TDH3-ILV3-HA	*ILV3*			p426TDH3	(2); this work
p416MET25-luc2	*luc2*	*MET25*	*CYC1 (258 bp)*	p416MET25 (2)	(9)
pCM182-HAP4	*HAP4*			pCM182 (3)	this work
pFET3-GFP	*GFP*	*FET3*	*CYC1*	p416MET25	(4)
p416MET25-hRluc	*hRluc*	*MET25*	*CYC1*	p416MET25	(9)
pMET25/LEU1-term	*luc2*	*MET25*	*LEU1 (477 bp)*	p416MET25-luc2	this work
pMET25-luc2	*luc2*	*MET25*	none	p416MET25-luc2	(9); this work,
pLEU1-luc2	*luc2*	*LEU1 (890 bp)*	*CYC1*	p416MET25-luc2	this work
pLEU1/LEU1-term	*luc2*	*LEU1 (890 bp)*	*LEU1 (477 bp)*	pLEU1-luc2	this work
$pLEU1^{P}$-600	*luc2*	*LEU1 (609 bp /BglII)*	*CYC1*	pLEU1-luc2	this work
$pLEU1^{P}$-230	*luc2*	*LEU1 (230 bp)*	*CYC1*	pLEU1-luc2	this work
$pLEU1^{P}$-175	*luc2*	*LEU1 (175 bp)*	*CYC1*	pLEU1-luc2	this work
$pLEU1^{P}$-175/LEU1-term	*luc2*	*LEU1 (175 bp)*	*LEU1*	$pLEU1^{P}$-175	this work
pCYC1-hRluc	*hRluc*	*CYC1 (385 bp /SmaI)*	*CYC1*	p416MET25-hRuc	this work
pCYC1-1kb-hRluc	*hRluc*	*CYC1 (1 kb)*	*CYC1*	p416MET25-hRuc	this work
pCYC1-luc2	*luc2*	*CYC1 (1 kb)*	*CYC1*	p414MET25-luc2	this work
pΔUAS1-hRluc	*hRluc*	*CYC1 (322 bp)*	*CYC1*	p416MET25-hRluc	this work
pΔUAS2-hRluc	*hRluc*	*CYC1 (385 bp; -309-247Δ)*	*CYC1*	p416MET25-hRluc	this work
pΔUAS1/2-hRluc	*hRluc*	*CYC1 (247 bp /XhoI)*	*CYC1*	p416MET25-hRluc	this work
pYES-HRG4	*CeHRG4*	*Gal-1*	*CYC1*	pYES-DEST52	(13)

Open reading frames for firefly luciferase, *luc2*, and Rhenilla luciferase, *hRLuc,* were taken from pGL3-basic and pGL-4.70 (Promega), respectively. Promoter fragments were directionally inserted into the *Sac*I and *Hind*III sites of p416MET25-luc2 or p416MET25-hRluc replacing the *MET25* promoter (9). For insertion of terminator fragments, the *Hpa*I and *Kpn*I sites flanking the *CYC1* terminator were used (9). The

number of base pairs indicate the size of the cloned fragments upstream of the ATG-codon (promoter) or downstream of the stop codon (terminator) of the respective genes. When endogenous restrictions sites were used for sub-cloning, the name of the enzymes are indicated.

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