J. Microbiol. Biotechnol. (2004), **14**(3), 470–473

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

High Copy Rme1p Suppresses Iron-Induced Cell Growth Defect of *Saccharomyces cerevisiae*

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Received: April 29, 2003
Accepted: June 13, 2003

Abstract In the yeast *Saccharomyces cerevisiae*, iron can be toxic. Because of this phenomenon, its metabolism of iron is strictly regulated. We have constructed a model system in which cell growth is defected during periods of iron overload. When Aft1-1^{up} protein was overexpressed with Gal10 promoter, a galactose inducible promoter, cell growth was defected and levels of *CLN2* transcript decreased. However transcript levels of *AFT1* and *FET3* genes increased over time in a consistent manner throughout the course of *AFT1-1^{up}* overexpression. We have screened to find genes to suppress cell growth defect by iron overload with YEp-derived high copy yeast genomic DNA library and found that high copy of Rme1p suppressed cell growth defects. Rme1p has been known as an activator protein of *CLN2* gene expression. Taking these results together, we suggest that the yeast cell cycle is arrested at the G_1 phase by iron overload via Cln2p.

Key words: Yeast, iron, oxidative stress, cell growth

Virtually all organisms need iron as an essential cofactor for diverse biological processes including the respiratory processes, and in oxidation-reduction [1, 10]. Although iron from the environment is not biologically widely available because of its low solubility, it becomes toxic to certain organisms when it is overloaded due to the generation of hydroxyl radicals *via* the so-called Fenton reaction [7]. Most organisms have a regulatory system controlling intracellular iron concentration. In *Saccharomyces cerevisiae*, iron metabolism is well characterized and there are two different pathways to take up free iron [6, 8]. They are high affinity pathways, exemplified by Ftr1/Fet3p, and low affinity pathways, exemplified by Fet4p. Most of the gene products involved in the high affinity pathway is regulated by the transcription factor protein Aft1p [15]. Aft1p binds upstream conserved regions and activates the transcription of the target gene [16]. The dominant mutant allele of *AFT1*, *AFT1-1^{up}*, was isolated by genetic screening and has been shown to have constitutively high expression levels [15]. There was a high level of iron uptake in the *AFT1-1^{up}* strain, which exhibited a high expression level of iron metabolism genes. This strain has also shown partial G_1 arrest when placed in high iron medium for Cln2p level [5, 11, 13]. To elucidate the iron-induced oxidative stress pathway, we have screened multicopy suppressor genes with a yeast genomic DNA library derived from YEp13 for a suppressor of growth defect by *AFT1-1^{up}* overexpression. We found that Rme1p acted as a suppressor. Rme1p is known to be a negative regulator of meiosis and activates the transcription of the *CLN2* gene [14]. In this study, we demonstrate the function of Rme1p as a suppressor during iron-induced oxidative stress.

Materials and Methods

Yeast Strains, Plasmids, and Media

The yeast strains used in this study are YPH499 [*MATa ura3-52 lys2-801 (amber) ade2-101 (ochre) trp1-63 his3-200 leu2-1*], and its derivatives. Yeast strains were grown in 1% yeast extract, 2% peptone, 2% glucose (YPD) or synthetic defined (SD) medium (6.7 g/l yeast nitrogen base) supplemented with any necessary auxotrophic requirements and either 2% glucose or 2% raffinose and 0.2% galactose for galactose induce medium. Transformation of yeast and *E. coli* was performed using standard methods [2]. For measuring the growth rate of each congenic strain, yeast was grown to mid-log phase in defined SD media, and then inoculated at an OD_{600} of 0.1 on selective media. Aliquots

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of cells were removed at regular intervals up to 18 h and the optical density (OD_{600}) was measured. The *AFT1-1^{up}* strain was cotransformed with pRS425, which is a yeast multicopy shuttle vector, and pGAF that contains the *AFT1-1^{up}*-coding sequence under the control of the *GAL10* promoter. PCR-mediated gene disruption was used to generate deletions of the *RME1* gene. The following primers were used to amplify the *HISG-URA3-HISG* cassette from the plasmid pMT604: for *RME1*, 5'-ACCGTGTTATGGACAAAACAGTGCCATCGCCAAGGGGTCTTGGAACAGAGCTCACTATAGGGCGAATTGG-3' and 5'-ATTTGGTATTGTTCAAAATTTTCTTCAAACGCTTGTTAAATCTTGAATCACTAAAGGGAACAAAAGCTGG-3'. Deletion was confirmed by PCR. Genomic clone of *CLN2* was constructed by isolating genomic DNA and digesting it with *Xba*I and *Pst*I. After electrophoresis, DNA fragments from 5.0- 5.5 kb pairs were extracted and ligated into *Xba*I and *Pst*I digested Yep351. Direct PCR from colonies was performed from 300 colonies and one genomic clone of *CLN2* was obtained.

Northern Blot Analysis

Cells were grown to mid-log phase in SD medium and total RNA was extracted using Trizol reagents (Life Technologies, Inc., St. Paul, MN, U.S.A.). Equal amounts (5 μg) of total RNA were analyzed by 1% formaldehyde-agarose gel electrophoresis and Northern blot was performed as previously described. Probes for internal regions of 0.2 kb of *CLN2*, 5'-TTTTCGAAGTATCGCACGGC-3' and 5'-ATGAAGGGTAGAACACCATTGACC-3'; for 0.2 kb of *ACT1*, 5'-ACACGGTATTGTCACCAACTGGG-3' and 5'-AGGACAAAACGGCTTGGATGG-3'; for 0.2 kb of *AFT1*, 5'-AGCATCCTCTTCAACTGTATCGTCC-3' and 5-CGTACCGGTCTTACGCAGCC-3'; for 0.2 kb of *FET3*, 5'-TTGGACGATTTCTACTTGCAACC-3' 5'-TCGGAGTTGTTTGCTTGATCACC-3' were used to be amplified by PCR.

RESULTS

Screening of High Copy Suppressor Genes

In the yeast *Saccharomyces cerevisiae*, Aft1p acts as a transcription factor in iron metabolism. Overexpression of the dominant mutant allele of *AFT1*, *AFT1-1^{up}*, results in cell cycle arrest at the G_1 phase. When *AFT1-1^{up}* was overexpressed by means of Gal10 promoter (a galactose inducible promoter), cell cycle arrest was induced by iron overload. We suggest this phenomenon contributes to iron overload since iron uptake was increased about 20-fold (personal communication with Dr. Caroline C. Philpott, NIDDK, NIH). Also, the iron-induced cell cycle arrest at the G_1 phase was caused by interference from G_1 cyclins [13]. We have constructed a screening method to understand

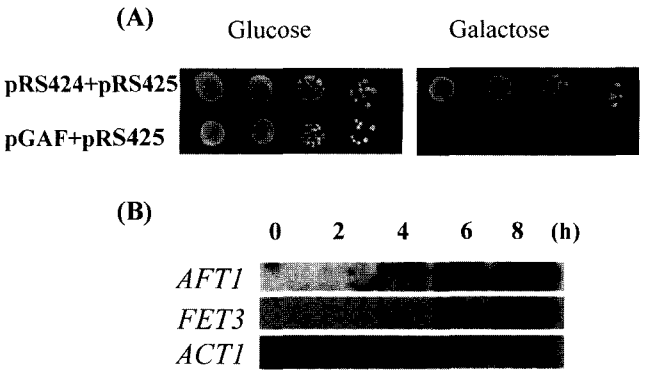

Fig. 1. *AFT1-1^{up}* overexpression results in the growth defect.
Congenic strains of the indicated genotype were plated in serial dilutions on synthetic SD medium and galactose-induced medium and incubated for 5 days at 30°C (A) and total RNA was isolated from *AFT1-1^{up}* overexpressed cells at indicated times from galactose induction. Northern blot analysis was performed with sequential hybridization of the indicated probe (B).

iron-induced cell cycle arrest in order to find suppressor genes, which suppress iron-induced cell cycle arrest, from a genomic DNA library. As shown in Fig. 1A, yeast cells overexpressed *AFT1-1^{up}* allele with a galactose-inducible promoter, and showed growth defect in contrast to the control strain. To confirm the expression level of the genes involved in iron metabolism, we performed Northern blot analysis of the *AFT1* and *FET3* genes and found that their transcript levels increased in correlation with the time course of *AFT1-1^{up}* expression (Fig. 1B). To screen suppressor genes, YEp13-based yeast genomic library and pGAF were cotransformed into YPH499 yeast strain and plated to -Trp -Leu SD plates. One day later, the transformants were collected and plated onto -Trp -Leu galactose induction media supplemented with 0.2% galactose

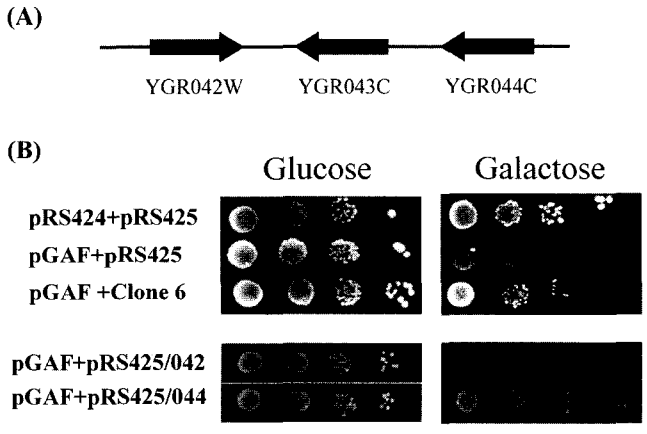

Fig. 2. High copy *RME1* suppresses iron-induced cell cycle arrest.
Clone 6 has three ORFs (A) and suppresses iron-induced growth defect (B). Congenic strains, which have indicated plasmids, were plated in serial dilutions on synthetic SD medium and galactose-induced medium and incubated for 5 days at 30°C (B).

and 2% raffinose. A total of 13 colonies appeared and plasmids were recovered from the yeast. The 13 inserts were identified and all had the same ORFs as YGR042w, YGR043c, and YGR044c (Fig. 2A). From Blast searches, inserts were identified, indicating that the YGR042w gene product has weak similarity to Troponin-I of *Clupea hareagus*, YGR043c has strong similarity to transaldolase, and YGR044c encodes the zinc-finger transcription factor Rme1p. Each ORF was cloned individually and confirmation was performed by plate assay. As shown in Fig. 2B, the strain, which has high copy of *RME1*, could suppress cell cycle arrest. Rme1p is known to act as a repressor of meiosis in the yeast *Saccharomyces cerevisiae*. It is also a zinc-finger protein, which regulates *CLN2* expression level [2, 3, 4, 9]. In addition, it blocks meiosis in haploid yeast cells in response to starvation by preventing the transcription of *IME1*.

High Copy Rme1p Suppresses *AFT1-1^{up}* Induced Growth Defect and Induces *CLN2* Transcripts

It has been previously reported that translation of Cln2p was regulated by overexpression of Aft1-1^{up}p rather than the transcript of *CLN2* [13] in *Saccharomyces cerevisiae*. To investigate the change of the *CLN2* transcript, we performed Northern blot analysis of *CLN2* in the Δ*rme1* and high copy RME1p strains. As shown in Fig. 3A, there was a high level of *CLN2* transcript but not in the Δ*rme1* strain in the high copy of RME1p strain. We also investigated the *CLN2* transcript level in the *AFT1-1^{up}* overexpression strain. Interestingly, we have found that *CLN2* transcript was decreased in the *AFT1-1^{up}* overexpression strain with time course from galactose induction (Fig. 3B). This result indicates that the transcript level of *CLN2* decreased as a result of overexpression of *AFT1-1^{up}*. We also tried to see whether or not overexpression of Cln2p suppresses the growth defect induced by *AFT1-1^{up}* overexpression. To obtain a genomic clone of the *CLN2* gene, genomic DNA was digested with *Xba*I and *Pst*I and about 5.2 kb fragments were subcloned into YEp351 yeast shuttle vector. *CLN2* genomic clone was screened by PCR. Genomic clone of *CLN2* and p*AFT1-1^{up}* was cotransformed and plate assay was performed with strains having high copy numbers of both the *CLN2* gene and p*AFT1-1^{up}*. Cells grown on SD selective medium were transferred to galactose inducible medium and incubated for 5 days. As shown in Fig. 3C, this strain, which has a high copy of *CLN2*, grew better than the control strain, but the difference was very small.

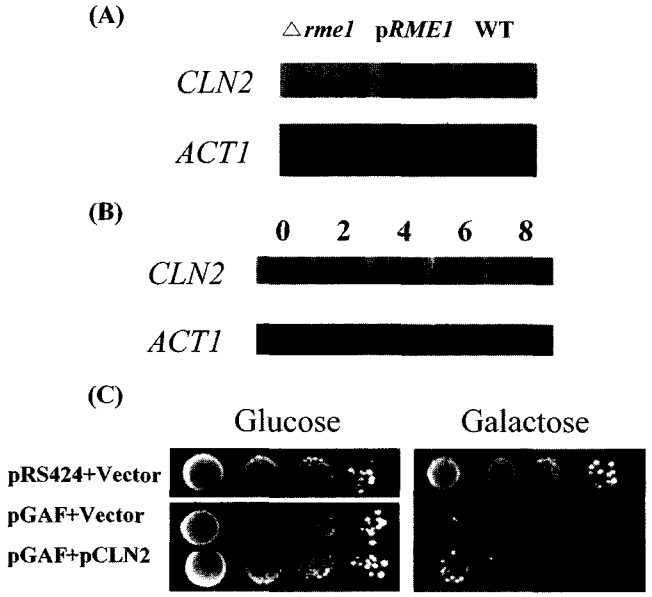

Fig. 3. Cln2p is involved in iron-induced cell growth defect.
Total RNA was isolated from indicated strains and Northern blot analysis was performed with sequential hybridization of the indicated probe (A). Total RNA was isolated from *AFT1-1^{up}* overexpressed cells with time course and Northern blot analysis was performed with sequential hybridization of the indicated probe (B). Overexpression of Cln2p partially suppresses iron-induced cell cycle defect. Congenic strains, which have indicated plasmids, were plated in serial dilutions on synthetic SD medium and galactose-induced medium and incubated for 5 days at 30°C (C).

DISCUSSION

Iron metabolism has been well studied in the yeast *Saccharomyces cerevisiae* as compared with this yeast in other organisms, and Aft1p has a pivotal function in iron metabolism. A lot of facts have been reported that iron overload induces cell cycle arrest at the G_1 phase in most organisms as a result of the formation of radicals [5, 11]. One interesting finding is that overexpression of Aft1p induces cell cycle arrest by inhibition of Cln2p translation rather than transcripts in yeast [13]. In the yeast *Saccharomyces cerevisiae*, the cell cycle is regulated strictly at the G_1 phase and G_1 cyclins as well as other proteins are involved in G_1-S transition [5]. Nutrients such as metal ion and sugars are also involved in G_1-S transition. We have found that the *CLN2* transcript level was decreased in the Aft1-1^{up}p overexpressed strain. Cln2p is one of the G_1 cyclins and maximum transcription is detected during the G_1 phase and decreases with cellcycle progression [5]. Strains with *CLN2* mutation are defected in cell cycle progress and the dominant mutant of *CLN2* advances the G_1-S transition because of its inability to halt at the G_1 phase [5]. Cln2p is involved in the regulation of cell growth and it depends on many factors including the availability of nutrients and is also regulated by environmental stresses. We have established the assay method to clarify the growth differences induced by Aft1-1^{up}p overexpression using *GAL10* promoter and performed screening to find high copy suppressor protein from the yeast genomic DNA library. High copy expression of Rme1p suppressed Aft1-1^{up}p induced cell growth defect

and activated the *CLN2* transcript. *RME1* transcripts are activated at the end of mitosis and in G_1, and Ace2p and Swi5p mediate *CLN2* expression [9]. Rme1p acts through two specific Rme1p response elements in the *CLN2* promoter to induce the expression of the *CLN2* gene [9]. We postulate that the *AFT1-1*^{up} strain takes up excess iron, which results in the inhibition of the G_1 phase mediated by Cln2p. In addition, we suggest that the overexpression of Rme1p suppresses the G_1 arrest caused by Aft1-1^{up}p overexpression with partial activity. Further study is required to find other factors in this iron-induced cell growth defect because high copy Cln2p partially suppresses the growth defect.

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