THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 21, Issue of May 23, pp. 13786–13792, 1997

Printed in U.S.A.

The Yeast Fre1p/Fre2p Cupric Reductases Facilitate Copper Uptake and Are Regulated by the Copper-modulated Mac1p Activator*

(Received for publication, July 18, 1996, and in revised form, February 11, 1997)

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Fre1p and Fre2p are ferric reductases which account for the total plasma membrane associated activity, a prerequisite for iron uptake, in *Saccharomyces cerevisiae.* The two genes are transcriptionally induced by iron depletion. In this communication, we provide evidence that Fre2p has also cupric reductase activity, as has been previously shown for Fre1p (Hassett, R., and Kosman, D.J. (1995) *J. Biol. Chem.* 270, 128–134). Both Fre1p and Fre2p enzymes are functionally significant for copper uptake, as monitored by the accumulation of the copper-regulated *CUP1* and *CTR1* mRNAs in *fre1*Δ, *fre2*Δ, and *fre1*Δ*fre2*Δ mutant strains. However, only Fre1p activity is induced by copper depletion, even in the presence of iron. This differential copper-dependent regulation of Fre1p and Fre2p is exerted at the transcriptional level of the two genes. We have shown that Mac1p, known to affect the basal levels of *FRE1* gene expression (Jungmann, J., Reins, H.-A., Lee, J., Romeo, A., Hassett, R., Kosman, D., and Jentsch, S. (1993) *EMBO J.* 12, 5051–5056), accounts for both the copper-dependent induction of *FRE1* and down-regulation of *FRE2* gene. Finally, Mac1p transcriptional activation function is itself modulated by the availability of copper.

Iron and copper are elements that have many similar chemical properties. Both are transition metals with several oxidation states, have close atomic and ionic radii numbers, and have very similar electronegativity. They are relatively homogeneously distributed on the planet and have thus been integrated in many biochemical reactions during evolution. Both are essential for life in almost every species (bacteria, fungi, plants, mammals) (1, 2). Excess quantities of iron and copper exert similar amount-dependent cytotoxicity, both favoring the formation of hydroxyl radicals which are disastrous to the cell (3). Iron presents a particular problem for its uptake, since in the oxygen-containing atmosphere it is practically all in the form of insoluble ferric hydroxides. Organisms have therefore developed complex mechanisms of high fidelity and precision to achieve an appropriate homeostasis of these two metals.

In *Saccharomyces cerevisiae,* two proteins of the plasma cell membrane, Fre1p and Fre2p, reduce Fe(III) to Fe(II) in the proximal vicinity of the cell. The expression of both corresponding genes is regulated by the environmental iron concentration by a negative feedback mechanism which takes place at the transcriptional level (4, 5). The coupled function of the cell surface Fet3p multicopper oxidase, which catalyzes the conversion of Fe(II) to Fe(III) extracellularly (6), and the recently reported Ftr1p permease (7) are also required for high affinity iron uptake. Thus, a link between iron and copper metabolism was first noted by the isolation of the *FET3* gene in a scheme aiming to clone the ferrous transporter (8). Fet3p requires copper to function, and therefore high affinity iron uptake requires copper (8, 9). Mutations either in the high affinity copper transporter gene *CTR1* or in the *CCC2* gene, encoding a member of the family of P-type ATPases proposed to transport cytosolic copper into the lumen of a secretory organelle, results in iron deficiency in the cell (9, 10). A similar mechanism has been postulated in mammals for the release of newly absorbed iron from intestine to blood involving the plasma glycoprotein ceruloplasmin, a copper-binding protein with ferrous oxidase activity (11, 12). Finally, the iron regulated transcription of the yeast genes *FRE1*, *FRE2*, *FET3*, *CCC2*, *FTR1*, and *FTH1* (*FTR1* homologue of unknown function) is affected by the Aft1p transcriptional activator which recognizes a specific consensus sequence on their promoters (13, 14). Moreover, expression of *FRE1* and *CTR1* mRNAs depends on the nuclear protein Mac1p, which is involved in iron and copper utilization (15).[1]

We have previously shown that although Fre1p and Fre2p have seemingly redundant functions in *S. cerevisiae*, the two genes are up-regulated by the absence of extracellular iron in a kinetically different way, implying that they are subject to distinct regulation (5). Since there is evidence that copper reduction might be a component of copper uptake (16) and that Fre1p is also a copper-repressible cupric reductase (15–17), in this study we have investigated the participation of *FRE1* and *FRE2* gene products in copper metabolism. Our data clearly point to a role of both activities in copper uptake, although the two genes are differentially affected by the function of Mac1p under conditions of copper (and iron) depletion. Mac1p transactivating function is itself modulated by the availability of copper, being higher in its absence.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—The yeast strains used in this study are all derivatives of the S288C strain. The wild type and *fre1*Δ, *fre2*Δ, and *fre1*Δ*fre2*Δ strains have been previously described (5). For the *MAC1* gene disruption in the yeast genome, a 480-base pair *Sty*I fragment (codons 41–201) was replaced by the 1.1-kilobase *Hin*dIII fragment of the *URA3* gene on a *MAC1* (−100 to 963)/pBluescript recombinant phagemid. The resulted insertion fragment was excised and used to transform the desired *ura3–52* strains (18). Uracil prototrophy was used for the selection of transformants. Transformants able to grow on glycerol only in the presence of 50 μM copper (15) were

* This work was supported by the Greek Ministry of Industry, Energy and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] E. Georgatsou, L. A. Mavrogiannis, G. S. Fragiadakis, and D. Alexandraki, unpublished observations.

further confirmed for the *MAC1* gene deletion by DNA blot hybridization. The yeast strain used for transcriptional induction and repression assays of the LexA-Mac1p fusion proteins to *lacZ* reporters was L9FT5, a derivative of FY105 (*MATα*, *his3*Δ::*LexA*op*HIS3 leu2*::*PET56 ura3–52 trp1*Δ63).

The growth media used were SD (2% glucose, 0.67% yeast nitrogen base; Difco) or SD supplemented either with 100 μM bathophenanthroline disulfonic acid-Na_2 salt (BPS) (Fe(II) and Cu(II) chelator) or with 100 μM bathocuproine disulfonic acid-Na_2 salt (BCS) (Cu(I) chelator) (16, 19, 20). For all experiments described, cells were grown to saturation in SD medium, subsequently resuspended in the same medium, and grown for 8–10 generations (exponential phase). They were then shifted to the desired medium (SD, SDBCS, or SDBPS) at a concentration of $4–5 \times 10^6$ cells/ml. Aliquots were removed from each culture every 2 h and assayed for copper and iron reducing activities simultaneously.

Genes and Plasmids—The *FRE2*/*lacZ* and *FRE1*/*lacZ* fusion plasmids were constructed starting from the low copy number *URA3*- containing YCP50 vector carrying a 6.3-kilobase *Bam*HI-*Sal*I fragment containing the *Escherichia coli lacZ* gene (except for the 8 amino-terminal codons of β-galactosidase), the *lacY* gene, and a portion of the *lacA* gene. The −977/+3 and −930/+3 promoter fragments of *FRE1* and *FRE2* genes, respectively, produced by polymerase chain reaction were cloned in the *Bam*HI site of the described plasmid in fusion to the β-galactosidase open reading frame.

The *MAC1* gene was isolated from a YCP50 yeast genomic library by the use of a synthetic oligonucleotide probe. The sequence of the oligonucleotide is 5′-GCCCTCTGATGCACGATGCACACGCA-3′. For the construction of the LexA-Mac1p fusion proteins, the *MAC1* gene sequence from +4 to +1249 nucleotides was synthesized by polymerase chain reaction and subcloned into the *Sma*I-*Kpn*I sites of plasmid YCp91, a derivative of pRS314 (*TRP1)* containing the *ADH1* promoter and 5′ untranslated region followed by an ATG codon and sequences encoding the SV40 nuclear localization signal, the HA1 epitope from influenza virus, and the LexA binding domain (21). The oligonucleotide sequences used for the polymerase chain reaction are 5′-TCCCCGGGATAATATTTAATGGGAACA-3′ and 5′-GGGGTACCTGAAGTGGTGGCATCGCTTA-3′. The reporter plasmids used for the transactivation and repressor assays were correspondingly pJK103, in which four *LexA* operators upstream of the *GAL1* TATA element control the *lacZ* gene transcription (21, 22), and pJK1621, in which four *LexA* operators, two upstream activation sequences, and TATA element of the *CYC1* gene control the *lacZ* gene transcription (23, 24).

The sequences of the antisense *CTR1*, *CUP1*, and *EF1α* synthetic oligonucleotides used as probes in the RNA blot hybridization analyses are 5′-CCTGCAACTTGGAATTCTCAAGGATGTC-3′, 5′-GTTACCGCAGGGGCATTTGTCGTCGTC-3′, and 5′-CCAGAATCGACATGACCGATAACGAC-3′, respectively.

End-labeling of these oligonucleotides with γ-[^{32}P]ATP was performed by DNA kinase (22). The described oligonucleotides were synthesized by the Institute of Molecular Biology and Biotechnology Microchemistry group (Heraklion, Crete, Greece).

RNA and DNA Blot Hybridization Analyses—Samples of total RNA and yeast DNA were prepared and blot-hybridized according to standard procedures (22).

Antisense RNA Probe Preparation—For the *FRE1* gene-specific probe, a *Pvu*II-*Sac*I genomic DNA fragment containing the coding region of the *FRE1* gene (−680 to +2280) was subcloned into the *Eco*RV site of the pBluescript II KS (+/-) phagemid (Stratagene). The recombinant plasmid was linearized by restriction at the *Spe*I site on the *FRE1* gene (+1893) and a 444-base (including polylinker sequences) single-stranded homogeneously radiolabeled antisense RNA probe was synthesized by the T3 RNA polymerase in the presence of α-[^{32}P]UTP (21). From the 444-base *FRE1* probe, a 298-base fragment was protected, which defined the 3′ end of the RNA 135 bases downstream of the translation termination. A protected doublet was observed with this probe due to three consecutive thymidines preceding this point, which created an unstable RNase digestion-prone A/T-rich region.

For the *FRE2* gene-specific probe, an *Mlu*I-*Afl*II genomic DNA fragment containing the coding region of the *FRE2* gene (−2041 to +2340) was subcloned into the *Eco*RV site of the phagemid described for the *FRE1*-specific probe. The recombinant plasmid was linearized by restriction at the *Aha*II site on the *FRE2* gene (+1682) and a 712-base probe was produced by the same procedure as for the *FRE1*-specific probe. From the 712-base *FRE2* probe, 654 bases were protected, *i.e.* the size of the whole probe omitting the polylinker sequences (the *FRE2* mRNA 3′ is downstream the *Afl*II site used for subcloning). A protected doublet was observed with this probe due to a region of seven consecutive thymidines preceding the *Afl*II site.

For the *HIS3* gene-specific probe, a *Hin*dIII genomic DNA fragment covering the coding region of the *HIS3* gene from +305 to +591 was subcloned into the *Hin*dIII site of the phagemid described for the *FRE1*-specific probe. The recombinant phagemid was linearized by restriction at the *Mse*I site (+312) and a 260-base probe was produced by transcription with the T7 RNA polymerase.

RNase A Protection Assays—10^5 cpm of each antisense RNA probe were simultaneously hybridized with 25 μg of total RNA in a 20-μl reaction mixture containing 75% formamide, 0.5 M NaCl, 10 mM Tris pH 7.5, and 1 mM EDTA overnight. The mixture was diluted in 300 μl of a solution containing 0.3 M NaCl and 5 mM EDTA and treated with RNase A (30 μg/ml) at 20 °C for 30 min, followed by treatment with proteinase K (300 μg/ml in 0.1% SDS) at 37 °C for 20 min. The mixture was phenol/chloroform extracted, ethanol precipitated, denatured at 92 °C for 5 min, and electrophoresed on a 40-cm, 6% polyacrylamide/bisacrylamide (19:1), 8.3 M urea-containing gel.

Reductase Assays—Ferric reduction activity was measured as described previously (5). Exactly the same conditions were used for the copper reduction assay, except that BCS and $CuCl_2$ was used instead of BPS and $FeCl_3$. For the Cu(I) quantification, an extinction coefficient of 12.25 mM^{-1} cm^{-1} was used to measure the Cu(I)BCS complex at 482 nm (A_{482}). The extinction coefficient used for Fe(II)BPS at 520 nm (A_{520}) was 22.39 mM^{-1} cm^{-1}.

β-Galactosidase Assays—β-Galactosidase activity assays (22) were performed following 7 h of growth in the desired medium.

RESULTS

Fre1p and Fre2p Both Are Cupric Reductases but Only Fre1p Is Induced by Copper Deprivation—It has been shown previously that Fre1p ferric reductase is also cupric reductase induced by copper depletion (16). In this report, we have examined Fre2p ferric reductase for cupric reducing activity assayed in a *fre1*Δ strain (5). We have first used iron depletion culture conditions known to induce Fre2p (5) to obtain measurable amounts of the enzyme. For comparison, we have assayed three additional strains, wild type, *fre2*Δ (Fre1p activity), and *fre1*Δ*fre2*Δ, in parallel according to the conditions described under "Experimental Procedures." The results presented in Table I show that Fre2p as well as Fre1p have cupric reductase activity.

Since we had previously observed that the two enzymes follow kinetically distinct induction by iron deprivation (5), we have assayed the same strains under conditions of copper depletion during 12 h of exponential growth. Our results (Table I) show that Fre1p was induced under copper depletion at similar levels as by iron depletion. In contrast, Fre2p was not detectably modulated by copper depletion. Fre2p activity was not induced even in the presence of the reducing agent sodium ascorbate (up to 500 μM), excluding the possibility of inefficient retention of the Cu(II) found in the minimal medium by the Cu(I)-specific chelator BCS (data not shown)). We have obtained the same results (activity observed only in the *fre2*Δ strain) by using medium reconstituted from its ingredients with the omission of copper (data not shown).

Data in Table I show residual copper-reducing activity in the *fre1*Δ*fre2*Δ strain which was not affected by metal depletion and accounted for about 80% of the activity in noninduction conditions. We have not detected similar residual ferric reducing activity in this *fre1*Δ*fre2*Δ strain (5) (Table I).

Although Fre2p did not show induction within 12 h in copper-depleted *fre1*Δ cultures, this was achieved later between 12 and 15 h in repeatedly diluted copper-depleting medium and remained relatively constant during the exponential growth of the cells (Fig. 1). We suggest that according to the proposed model of iron-copper connection (8, 9) prolonged depletion of copper intracellularly created also iron depletion, which in turn induced Fre2p activity.

Copper Reduction by Fre1p and Fre2p Cupric Reductases Facilitates Copper Uptake—While our results showed that both Fre1p and Fre2p are cupric reductases, they do not an-

TABLE I

Fre1p and Fre2p induction under copper and iron depletion

Assays of reductase activities were performed in aliquots of the indicated strain cultures at different time points of growth in minimal (SD), copper-depleted (SDBCS), and iron-depleted (SDBPS) media. The average unit values (multiplied by a factor of 10) from five identical experiments obtained at 4 and 10 h of growth (when peak activities of the two reductases were detected) are shown. Units are defined as Cu(I) or Fe(II) nmol/h/10^6 cells.

Medium	Strain	Cupric reductase units		Ferric reductase units	
		4 h	10 h	4 h	10 h
SD	WT	5.3 ± 0.8	6.3 ± 2.0	4.5 ± 1.3	5.5 ± 2.7
	*fre1*Δ	5.0 ± 1.8	4.8 ± 0.4	1.2 ± 1.0	1.4 ± 0.9
	*fre2*Δ	4.2 ± 1.0	4.6 ± 0.5	6.1 ± 3.5	4.2 ± 1.9
	*fre1*Δ*fre2*Δ	4.7 ± 2.0	3.6 ± 0.3	0.8 ± 1.4	1.4 ± 1.1
SDBCS	WT	**38.5 ± 4.5**	**18.8 ± 1.8**	**102.5 ± 26.1**	**48.8 ± 7.1**
	*fre1*Δ	4.2 ± 0.0	5.3 ± 1.3	1.9 ± 1.8	3.4 ± 1.8
	*fre2*Δ	**35.5 ± 5.1**	**15.8 ± 1.8**	**104.6 ± 15.9**	**45.8 ± 4.8**
	*fre1*Δ*fre2*Δ	4.2 ± 0.4	4.7 ± 0.6	2.0 ± 1.7	3.2 ± 1.0
SDBPS	WT	**40.2 ± 3.8**	**47.0 ± 2.0**	**120.7 ± 35.0**	**91.2 ± 31.8**
	*fre1*Δ	4.4 ± 2.7	**32.0 ± 1.1**	1.4 ± 1.4	**53.6 ± 17.0**
	*fre2*Δ	**31.8 ± 7.9**	**12.0 ± 4.1**	**103.7 ± 20.9**	**33.6 ± 18.2**
	*fre1*Δ*fre2*Δ	5.2 ± 0.7	3.8 ± 0.1	1.8 ± 1.6	1.8 ± 1.3

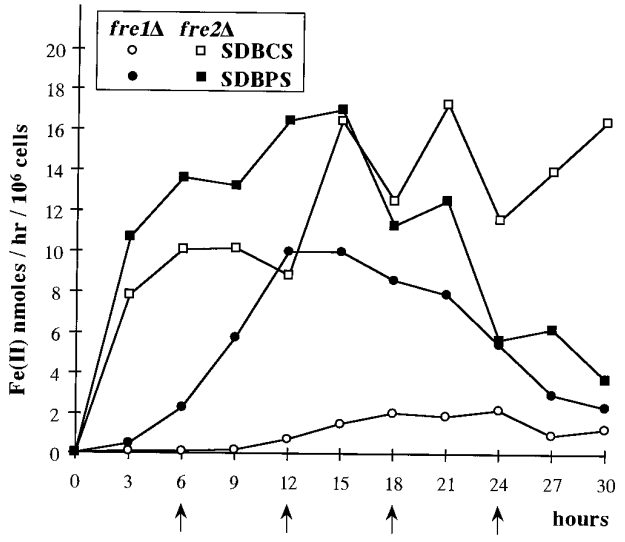

FIG. 1. **Induction of Fre1p/Fre2p reductases during prolonged growth in copper- and iron-depleted media.** Ferric reductase assays were performed in *fre1*Δ and *fre2*Δ strains during growth in SDBCS and SDBPS media. Ferric reductase activity was assayed in preference to cupric reductase activity to avoid the interference of the residual noninduced activity and to obtain higher values. The assay and growth conditions were as described under "Experimental Procedures," except that the cultures were diluted in fresh medium to a density of 4.5×10^6 cells/ml at 6-h intervals (*arrows*) to avoid saturation. The reductase assays for these particular time points were performed immediately prior to dilution. The results of one of two identical experiments are shown.

swer the question of whether copper reduction is necessary for copper uptake. Assuming that Fre1p and Fre2p are indeed important for copper uptake, this process should be impaired in a *fre1*Δ*fre2*Δ strain. We have monitored copper entrance into the cell and its utilization by following the expression of two different copper responsive genes, *CUP1* and *CTR1*. The regulation of these genes by copper follows distinct pathways. *CUP1* transcript encoding a metallothionein is induced by the transcription factor Ace1p when copper concentration increases in the cell (25, 26), whereas *CTR1* transcript encoding the copper transporter is induced by copper depletion and is not affected by the Ace1p factor (27). As shown in Fig. 2*A*, high levels of *CTR1* mRNA were detected in *fre1*Δ*fre2*Δ cells grown in minimal medium (SD), which indicated that the mutant cells had lower copper uptake capacity than the wild type cells. Nonetheless, addition of copper to a concentration of 0.5 μM completely repressed *CTR1* mRNA levels in both strains, indicating that the metal could enter also the *fre1*Δ*fre2*Δ cells. No significant difference was detected in the accumulation of *CUP1* mRNA between the two strains, possibly because *CTR1* gene responded to lower levels of copper than *CUP1* gene (27).

When cultures grew under Fe(II) and Cu(II) retention conditions (SDBPS) when both reductases were induced, prominent differences in the accumulation of both *CTR1* and *CUP1* mRNAs were obtained between *fre1*Δ*fre2*Δ and wild type strain. One hundredfold higher $CuSO_4$ concentration was necessary to diminish *CTR1* mRNA levels in the mutant cells. This observation demonstrated that the two reductases were clearly required for copper uptake under conditions of strong retention by chelators. As expected, when the two cultures grew under Cu(I) depletion conditions (SDPBCS), the difference between the two strains almost disappeared, since copper chelation was exerted after the step of reduction. The small remaining difference might reflect a higher Cu(I) concentration in the presence of reductases facilitating the Cu(I) transporter to compete with the chelator.

To evaluate the contribution of each of the reductases for copper uptake, we have similarly tested the singly disrupted strains *fre1*Δ and *fre2*Δ under conditions at which the double disruptant and wild type cells had shown the most prominent differences (SDBPS) (Fig. 2*B*). Comparison of the extent of *CTR1* mRNA repression in the four strains showed clearly that both reductases participated in copper uptake. The similarity of *CTR1* mRNA patterns between *fre2*Δ and wild type cells implied that Fre1p carried through the process almost as well as both reductases. However, a clear-cut difference between the *fre1*Δ and *fre1*Δ*fre2*Δ strains (a 5-fold $CuSO_4$ concentration was required to repress *CTR1* mRNA in the doubly disrupted cells) was detected, attributing to the Fre2p minor participation in copper uptake. The detected *CUP1* mRNA accumulation could not distinguish in either case between the presence or absence of Fre2p, but clearly pointed to the important contribution of Fre1p.

The Availability of Copper Affects the Transcription of FRE1 and FRE2 Genes—The differential induction profiles of the two reductases in copper depleted cells led us to investigate the levels of *FRE1* and *FRE2* mRNAs under these conditions. Using an RNase A protection assay we were able to simultaneously detect and quantitatively compare *FRE1*, *FRE2*, and *HIS3* (as an internal control) mRNAs in total RNA of a wild type strain. Fig. 3*A* (*left panels*) shows that *FRE1* mRNA accumulated under copper depletion and *FRE2* mRNA was barely detectable (only at very long film exposures) at all time points, as described for the induction of the corresponding

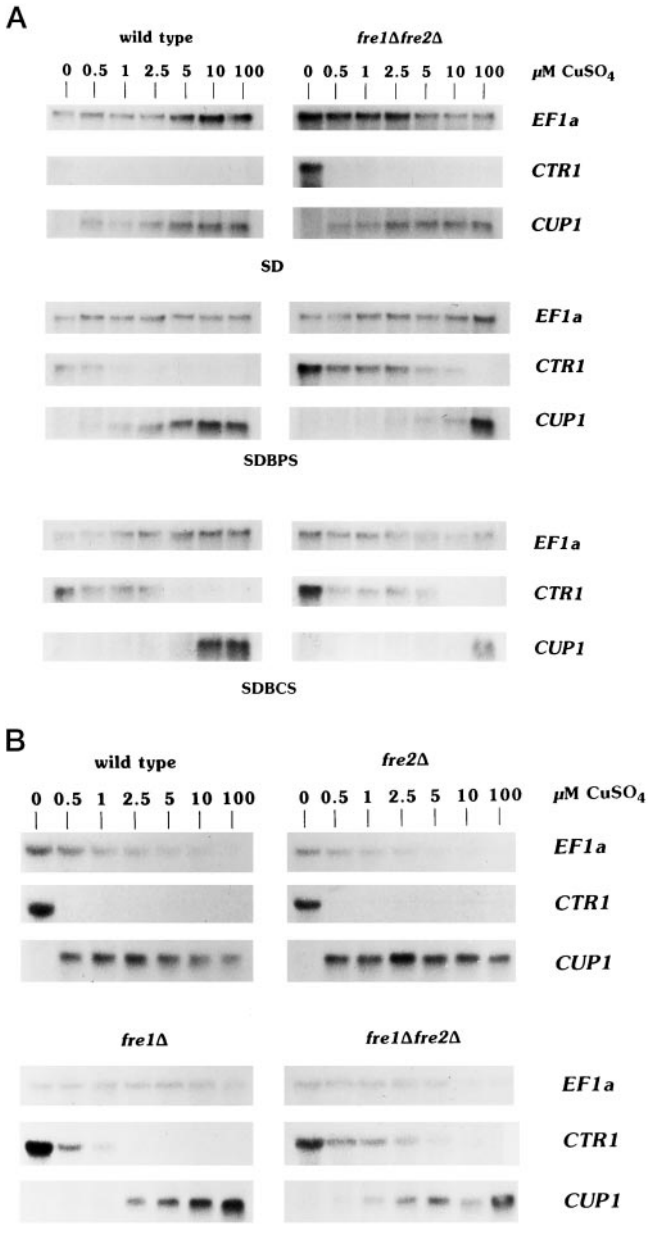

FIG. 2. **Uptake and utilization of copper monitored by *CTR1* and *CUP1* mRNA accumulation.** *A,* wild type and *fre1Δfre2Δ* cultures were first grown in SD, SDBPS, or SDBCS medium for 7 h. Aliquots were subsequently incubated in the presence of the indicated quantities of $CuSO_4$ (0.5–100 μM final concentration on top of the 0.25 μM contained in the SD medium) at 30 °C for 5 min. Fifteen μg of total RNA were prepared and analyzed by RNA blot hybridization using the radiolabeled *CTR1*, *CUP1*, and *EF1α* (*EF1a* on figure) oligonucleotide probes as described under "Experimental Procedures." *EF1α* (translation elongation factor 1 subunit α) probe was used as an internal control of quantitatively unaffected specific RNA accumulation. *B,* the mRNA patterns of wild type, *fre1Δ*, *fre2Δ*, and *fre1Δfre2Δ* cells grown in SDBPS, incubated in the presence of the indicated amounts of $CuSO_4$ at 30 °C for 10 min, and treated as described in *A*.

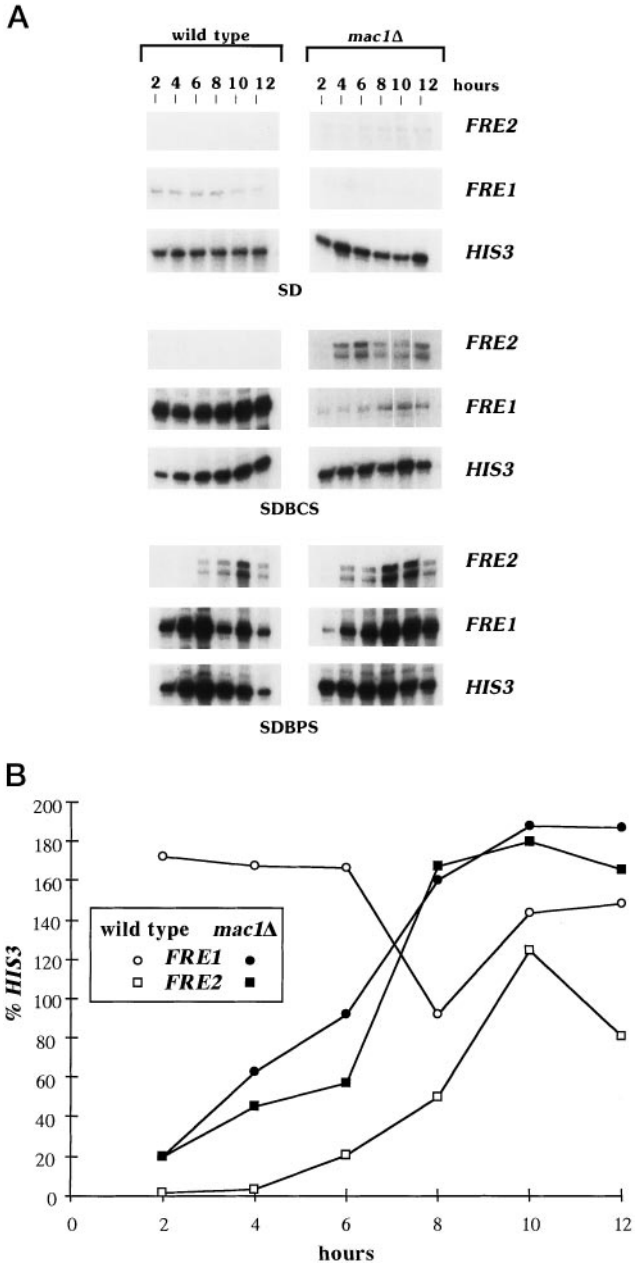

FIG. 3. **Copper- and iron-dependent *FRE1* and *FRE2* mRNA accumulation in wild type and *mac1Δ* strains.** *A,* total RNA prepared during growth of wild type and *mac1Δ* strains in minimal SD, SDBCS, or SDBPS medium was subjected to RNase A protection assay by the indicated (*right*) radiolabeled antisense RNA probes and analyzed by gel electrophoresis and autoradiography as described under "Experimental Procedures." The *HIS3* antisense RNA probe was used as an internal control of quantitatively unaffected specific RNA accumulation. *B,* densitometric analysis of the lanes in SDBPS panels (*wild type* and *mac1Δ*) shown in *A*. The relative values of *FRE1* and *FRE2* mRNAs are expressed as the percentage of the *HIS3* mRNA value in each lane.

enzymatic activities. In parallel experiments using iron-depleted cultures, *FRE1* and *FRE2* mRNAs accumulated both following the time-dependent induction observed for the corresponding reductase activities (5) (Table I).

Measurements of β-galactosidase activities driven by the *FRE1* or *FRE2* promoters in wild type cells grown under copper and iron depletion indicated clearly that the *FRE1* promoter was able to confer both copper- and iron-regulated expression of β-galactosidase, whereas the *FRE2* promoter conferred only iron-regulated expression (Fig. 4).

The Function of Mac1p Accounts for the Differential Copper-dependent Regulation of FRE1 and FRE2 Gene Expression—The only known nuclear protein implicated in both copper and iron metabolism, affecting basal expression of the *FRE1* gene is Mac1p (15). *mac1* mutant cells suffer from copper and, possibly, iron deficiency, since addition of copper or iron (partly) can complement their phenotypes (15). Mac1p contains a region homologous to the copper-dependent transcription factor Ace1p (15). We have followed the response of *FRE1* and *FRE2* genes to copper depletion in a *MAC1* gene-deleted strain during 12 h

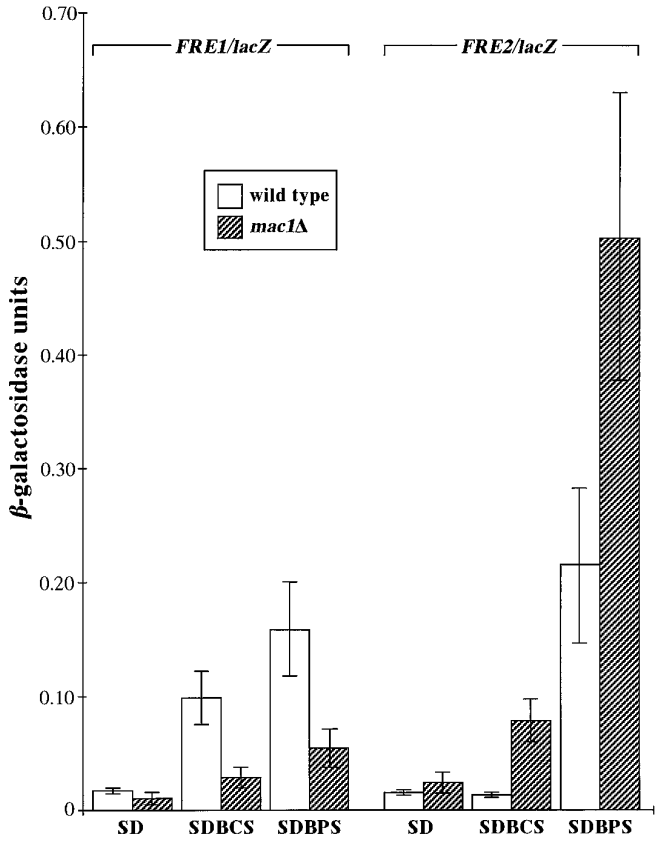

FIG. 4. **FRE1 and *FRE2* promoter-driven β-galactosidase activity in wild type and *mac1*Δ strain.** β-Galactosidase activities measured from cultures of wild type (*open bars*) or *mac1*Δ (*hatched bars*) strains transformed with the chimeric plasmids *FRE1/lacZ* and *FRE2/lacZ* and grown for 7 h in minimal (*SD*), copper-depleted (*SDBCS*), and iron-depleted (*SDBPS*) media.

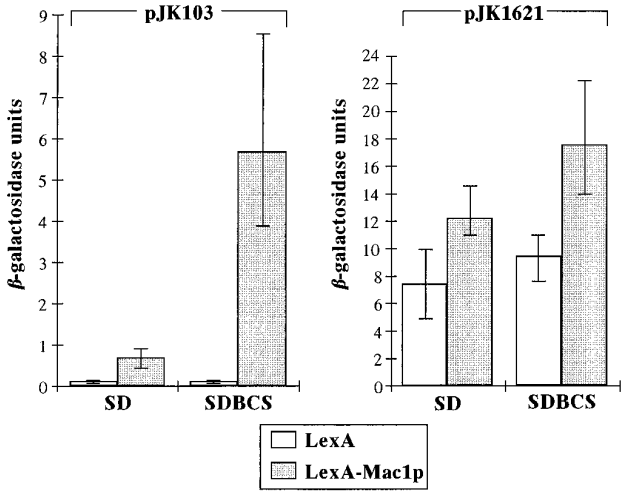

FIG. 5. **Copper-modulated transcriptional regulation by LexA-Mac1p.** β-Galactosidase activities obtained from pJK103 or pJK1621 *lacZ* reporters co-transformed with plasmids YCp91 (LexA) or YCp91-Mac1p (LexA-Mac1p fusion) as described under "Experimental Procedures". Measurements were performed in L9FT5 transformants grown for 7 h in minimal (*SD*) and copper-depleted (*SDBCS*) media.

of growth. The results shown in Fig. 3*A* (*right panels*) revealed that *FRE2* mRNA was detectable in *mac1*Δ cells grown in minimal medium (SD) and substantially induced following copper depletion. In contrast, *FRE1* mRNA basal levels were significantly lower in the *mac1*Δ uninduced cells (SD medium) compared with the wild type levels and accumulated at very reduced levels in the copper-depleted cells. Following iron depletion of the *mac1*Δ strain, both *FRE1* and *FRE2* mRNAs were induced. However, by comparison to the induction patterns obtained in wild type cells, we found a temporal shift of the *FRE1* mRNA induction to later time points in the exponentially growing *mac1*Δ culture, in contrast to a temporal shift of the *FRE2* mRNA accumulation to earlier time points (Fig. 3, *A* and *B*).

β-Galactosidase activity levels produced by the *FRE1/lacZ* and *FRE2/lacZ* plasmids in a *mac1*Δ strain grown under copper or iron depletion conditions showed that the differential accumulation of *FRE1* and *FRE2* mRNAs resulted from the transcriptional response of the two genes (Fig. 4). Comparing these results to the RNA accumulation profiles, we should note that at 7 h of growth (when the assays were performed) the accumulation of β-galactosidase was less in the *mac1*Δ strain compared with the wild type strain when driven by the *FRE1* promoter and more when driven by the *FRE2* promoter. The results of reductase assays on copper- and iron-depleted *fre1*Δ*mac1*Δ and *fre2*Δ*mac1*Δ cultures were in agreement with the induction patterns of their transcripts (data not shown).

Therefore, under copper limitation, Mac1p function has a negative role on *FRE2* gene regulation, while it affects positively *FRE1* gene. In the absence of Mac1p (in *mac1*Δ strain) under copper limitation, additional inducing factors might exist for both genes, since *FRE2* and *FRE1* mRNA levels are higher than those detected in the noninducing culture (minimal medium). Furthermore, as presented in the diagrams shown in Fig. 3*B,* Mac1p seems to be involved in the temporally differential expression of *FRE1/FRE2* genes under iron limitation. These observations are elaborated further under "Discussion."

Mac1p Transactivating Activity Is Modulated by Copper—The observed copper-dependent regulation of *FRE1/FRE2* genes affected by Mac1p function led us to investigate the copper dependence of Mac1p protein function. First we have tested the entire Mac1p coding sequence fused to the LexA binding domain for its ability to activate transcription of a β-galactosidase reporter following binding to the LexA operator (21, 22). As shown in Fig. 5, Mac1p protein was capable of transactivating the β-galactosidase reporter in cells grown in minimal medium. This activity was significantly increased when cells were depleted from copper, which suggests that the Mac1p protein was activated at these conditions. Mac1p mRNA levels were unaffected under copper-depleting conditions (data not shown). These results implicate a direct effect of copper on Mac1p modulating its transcriptional activation function. Testing of the LexA-Mac1p fusion for repression function on a *CYC1* promoter-driven *lacZ* transcription (23, 24) showed inducing rather than repressing effect on the constitutive expression of this reporter (Fig. 5), indicating that Mac1p probably does not have repressing activity. This result is relevant for the explanation given for its different role on the expression of the *FRE1/2* genes (see under "Discussion").

DISCUSSION

Iron and copper are metals whose biological importance and common properties have been emphasized frequently. The cellular mechanisms by which living organisms exploit them to fulfill their needs and simultaneously protect themselves are now being unravelled (12). The externally directed enzymes Fre1p and Fre2p are situated at the "beginning" of the pathway that links extracellular to intracellular iron communication, leading to nuclear events that alter gene expression, as well as at the "end" of such a pathway, being themselves regulated by iron to control its influx into the cell. Although a common iron-related transactivator, Aft1p, has been identified (13, 14),

the two reductase genes are differentially induced (Ref. 5 and this paper).

In this study we have examined the participation of the ferric reductases Fre1p and Fre2p in copper metabolism, and we have revealed elements of their metal-regulated expression. Previous reports have presented the ferrireductase plasma membrane activity (17) and Fre1p (16) as being repressed by copper. We have demonstrated that Fre2p is a cupric reductase, as is Fre1p, but only Fre1p is induced in copper starvation, accounting for 80–90% of the plasma membrane activity. The residual copper-specific reducing activity, which is not modulated by copper, probably corresponds to the Cu(II)-specific reductase, not repressible by iron, described by Hassett and Kosman (16). This reductase is possibly encoded by another *FRE*-homologous gene.[2]

We have further shown that the distinct response of the two cupric reductases to copper depletion is reflected from the differential transcriptional regulation of the two genes. While *FRE1* gene was transcriptionally up-regulated, *FRE2* gene did not seem to respond to copper depletion. Furthermore, we have shown that Mac1p nuclear protein contributed directly or indirectly to this differential response of the two reductase genes. *FRE1* gene basal and induced expression were highly dependent on its presence, in agreement with the notion that Mac1p is involved in the communication of the copper starvation signal to the *FRE1* gene. In contrast, *FRE2* gene basal and copper-induced expression were only observed in the absence of Mac1p. An explanation for this phenomenon, which needs to be proven, may lie in the following observations. *FRE1* promoter contains a pyrimidine-rich directly repeated sequence, previously shown to mediate iron-regulated transcription of a reporter gene (4), distinct from the Aft1p binding consensus, which could be responsible for the Mac1p effects, as discussed by Yamaguchi-Iwai *et al.* (14). Similar pyrimidine-rich repeated sequences are also found on the *FRE2* (5) and *CTR1* (9) promoters in different orientations. *CTR1* gene expression is also dependent on the presence of Mac1p (15).[1] Only on the *FRE2* promoter are these sequences situated next to the TATA box between positions −54 and −80. (The transcription start site of *FRE2* gene is at position −5 from the first coding AUG[1] and the potential TATA box is at position −89.) If these sequences were indeed affected by Mac1p, they might play different roles on the different promoters. While affecting positively the expression of *FRE1* and *CTR1* genes, Mac1p may result in steric hindrance of the formation of the transcriptional complex on the *FRE2* promoter (28). This hypothesis is also in agreement with our results indicating that Mac1p plays a major role in the temporally different induction of the two reductase genes by the use of the Fe/Cu chelator (BPS medium). We can conceive that upon metal deprivation Mac1p facilitates *FRE1* expression and inhibits *FRE2* expression. Accumulation of Aft1p and/or appearance of another epistatic *FRE2*-specific inducer results in the *FRE2* late induction.

This scheme is in agreement with our additional findings on the role of Mac1p. First, Mac1p was able to transactivate a reporter gene (*lacZ*) when artificially brought to its promoter (by the LexA binding domain). Second, Mac1p did not show any repression function on a constitutively expressed reporter gene (*CYC1*(UAS)-*lacZ*). Third, an important finding that directly related Mac1p function to the cupric reductases was the fact that its transactivation function was modulated by the availability of copper, being increased in its absence. The precise role of Mac1p will be revealed by the detailed study of its functional domains as well as examination of the interacting elements of the transcriptional complexes in which it participates.

Our last finding concerns the role of the Fre1p/Fre2p cupric reductases on the uptake of copper. Asking the question whether copper reduction is necessary for its cellular uptake, the profit from such a reaction is not immediately obvious since Cu(II) ions, unlike Fe(III), are found in a soluble form in water-dominated environments. Evidence as to whether copper enters the cell as Cu(II) or Cu(I) was indirect. Several studies in plants have shown a concomitant increase in Fe(III) and Cu(II) reducing activities in response to depletion of these metals from the soil (29, 30). These observations could be explained either by introducing the notion of a general role of reductases in regulating the redox state of the plasma membrane which would affect channel gating and ion influx (17, 30) or a more specific role in copper uptake which would take into account environmental factors, *e.g.* the availability of Cu(II) ions which form very stable complexes with various environmental ligands. Hassett and Kosman (16) presented evidence for copper reductive assimilation in *S. cerevisiae,* since Pt inhibition (Fre1p inhibitor) of copper uptake was relieved when the reducing agent ascorbate was included in the uptake assay. Very recently, Knight *et al.* showed that *FRE1* gene is necessary for the Ctr1p and Ctr3p function in ^{64}Cu uptake (31).

We have obtained evidence for the importance of reduction for copper uptake by using the *CTR1* and *CUP1* mRNAs as reporters to monitor entrance and utilization of copper in the cell. We have demonstrated that Fre1p/Fre2p activities assure higher intracellular copper levels, as shown by the increased *CTR1* mRNA quantities in the *fre1*Δ*fre2*Δ strain compared with wild type cells. This activity was not an absolute prerequisite for copper uptake, possibly due to the entrance of Cu(II) ions or to Cu(I) produced by the residual plasma membrane copper-reducing activity mentioned above. Our results provide evidence that mostly Fre1p but also Fre2p have a major contribution to the uptake of copper when we used a chelator capturing its oxidized form (BPS). This contribution might be very useful for the organism, since there are probably many chelators of Cu(II) in the ecosystem of *S. cerevisiae* (*e.g.* citrate in fruits). The involvement of the two ferric reductases in copper and iron metabolism appears distinct and specific, particularly since Fre1p and Fre2p do not respond to molybdenum, zinc, or manganese deprivation.[1] In reference to the iron-copper connection already discussed, it seems inevitable that ferric reductases have evolved to participate also in copper metabolism. Since iron cannot enter the cells in conditions of copper starvation, induction of the ferric reductases by iron depletion would not be of any use if copper entrance was not facilitated simultaneously. On the other hand, when copper is depleted, Fre1p is first induced, able to assume most part of copper import into the cells, and if, for some reason, copper continues to be rare, Fre2p comes to its aid, being induced much later indirectly because of iron deprivation. A clear contribution of Fre2p was seen both under prolonged copper starvation and in the *mac1*Δ strain. Our results show clearly that reduction of copper facilitates its entrance into the cells. We propose that Fre1p and Fre2p, although not indispensable for copper uptake, are actively and catalytically participating in that process, especially under natural conditions.

In conclusion, the Fre1p/Fre2p system, involving two genes distally related in primary structure (5) functioning in close collaboration for iron and copper handling but with clearly differentiated controlling mediators, is a very promising experimental model in revealing "fine tuning" mechanisms that have evolved in living organisms to assure profitable metal homeo-

[2] Internet data bases (http://speedy.mips.biochem.mpg.de/mips/yeast and http://genome-www.stanford.edu/Saccharomyces).

stasis. It is interesting to note that, as revealed by the completed sequence of the *S. cerevisiae* genome,[2] six additional open reading frames share similarities with the Fre1p and Fre2p sequences and possibly are involved in other specific membrane reductase activity functions.

Acknowledgments—We thank George Thireos and Dimitris Tzamarias for helpful suggestions; Apostolis Klinakis, Alexis Zafiropoulos, Thanassis Tartas, and Alexandra Boutla for help with plasmid and strain constructions; Georgia Houlaki for artistic assistance; and Lila Kalogeraki for photographic assistance.

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